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## Anti-sperm Autoantibody Inhibits Fertility of Spermatozoa in the Nile Tilapia, *Oreochromis niloticus*

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### Abstract

In order to investigate whether sperm surface autoantigens are involved in fertilization, the fertility of sperm coated with anti-sperm autoantibodies was examined in the Nile tilapia, *Oreochromis niloticus*. Anti-sperm autoantibodies were isolated from serum of male Nile tilapia immunized with allogeneic sperm emulsified with Freund's complete adjuvant. Freshly spawned eggs were inseminated with sperm treated either with autoantibodies or with non-specific antibodies diluted in a graded series with artificial seminal plasma. The fertility of sperm coated with autoantibodies was prominently lower than that of sperm coated with non-specific antibodies, and the inhibition of fertility was apparently dependent on the concentration of the antibodies. Coating with antibodies did not affect sperm motility. Some sperm agglutinations were observed at the highest dilution of autoantibodies, but the agglutination occurred fairly gradually and did not prevent the spermatozoa to fertilize the egg. These results suggest that at least one of the autoantigens existing on sperm surface may play an important role in fertilization in the Nile tilapia.

### Introduction

In the testis of fishes, several specific proteins are expressed on the sperm plasma membrane during spermiogenesis (Parmentier et al., 1984; Lou and Takahashi, 1991). These proteins are known as autoantigens which are isolated from the fish's own immune system by the blood-testis barrier (Lou and Takahashi, 1989), and may have specific functions during and after spermiogenesis. Lou and Takahashi (1991) identified sperm-surface autoantigens from the isolated sperm plasma membrane of Nile tilapia, *Oreochromis niloticus*. The results showed that the tilapia sperm had at least six kinds of autoantigens on their surface. It is very interesting to know the physiological significance of the autoantigens.

In mammals, it is well known that sperm-specific antigens play an important role in fertilization processes, such as sperm zona pellucida binding (Yanagimachi et al., 1981; Bronson et al., 1982; Moore and Hartman, 1984; Myles et al., 1987) and sperm-egg membrane fusion (Primakoff et al., 1987).

Although spermatozoa of fishes have no acrosome, in sharp contrast to those of mammals, it is possible that autoantigens on fish sperm may function in a similar manner to those on mammalian sperm. In the present study, in order to investigate whether the sperm-surface autoantigens are involved in fertilization, the fertility of spermatozoa coated with an anti-sperm autoantibody was examined in the Nile tilapia.

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## Materials and Methods

### *Animal*

Mature tilapia, *Oreochromis niloticus*, of both sexes used in the present study were maintained in indoor concrete ponds on the campus of the Faculty of Fisheries, Hokkaido University, at 25-30°C under natural light conditions, and fed on a commercial diet for trout culture (Nippai Fish Food Co.) 2 to 3 times a day.

For artificial fertilization, mature female fish were transferred to a 60-liter glass aquaria with well-aerated and filtrated water regulated at  $28 \pm 2^\circ\text{C}$  by an electric heater.

### *Antibody purification*

Anti-sperm autoantibody was purified from Nile tilapia anti-sperm autoantiserum which was obtained by the method described by Lou et al. (1989). Non-specific tilapia IgMs were isolated and purified by the same method from sera of the intact tilapia.

The "autoantibody solution" and "non-specific antibody solution" were prepared for some experiments of the present study. The purified anti-sperm autoantibody was adjusted to 6 of the sperm-agglutination titer (Lou and Takahashi, 1988), and was diluted serially to  $2^{-3}$ ,  $2^{-6}$  and  $2^{-9}$ . The non-specific antibody was also diluted to the same protein concentrations. Protein determinations were carried out according to the method described by Bradford (1976).

### *Assessment of sperm fertility*

Milt, which was stripped freshly from mature male tilapia, was suspended in artificial seminal plasma (ASP) (Mochida, 1992), and adjusted to  $10^7$  cells/ml. One hundred  $\mu\text{l}$  of the sperm suspension was mixed with an equal volume of each of the autoantibody solutions and non-specific antibody solutions which had previously been dialyzed against ASP overnight. After incubation for about 4-6 h at room temperature, each of the autoantibody- and non-specific antibody-sperm mixtures was diluted in 5 ml of water and mixed with a group of eggs stripped from a female fish. After settlement for 30-40 min, the eggs were transferred to the incubator for Nile tilapia eggs. Two days after artificial fertilization, fertility was measured by counting the eggs displaying pigmentation. In each antibody dilution group, the total number of eggs counted was about 2,000 and the experiments were repeated four to five times.

At the time of insemination, the motility of the sperm, which had reacted to a series of antibodies, was measured. Twenty  $\mu\text{l}$  of the sperm-antibody solution were diluted with 200  $\mu\text{l}$  of water on a slide glass. Sperm motility was determined by measuring the duration until all spermatozoa ceased to move in five different fields observed with a light microscope of  $100\times$  magnification.

A sperm-agglutination test was carried out on the sperm immediately after the determination of their motility. The test was to count the number of masses of agglutinated sperm in five different fields at a magnification of 100 diameters.

The percentage of spermatozoa bound to antibodies was also examined. A series of the sperm-antibody mixtures diluted with water as described above were dropped onto slide glasses coated with 0.01% poly-L-lysine. After 3-h settlement, non-specific binding sites were blocked with 10% normal rabbit serum for 1 h at room temperature. After being incubated with rabbit anti-tilapia IgM antiserum, which was obtained by the method described by Lou et al. (1989), for 1 h at room temperature, the specimens were incubated with FITC-conjugated goat anti-rabbit IgG antibodies (Sigma, dilution 1 : 80) for 1 h at room temperature in a dark humid chamber. The slide was observed under an Olympus BH microscope equipped with epifluorescence.

Sperm cells at four to five random locations on each slide were photographed, and the resultant photographs were used to calculate both the total number of sperm and the number of sperm that bound autoantibodies on their surface in each location.

*Statistical analysis*

In the present study, results were expressed as mean $\pm$ SE, and differences between groups were compared by paired Student's *t* test.

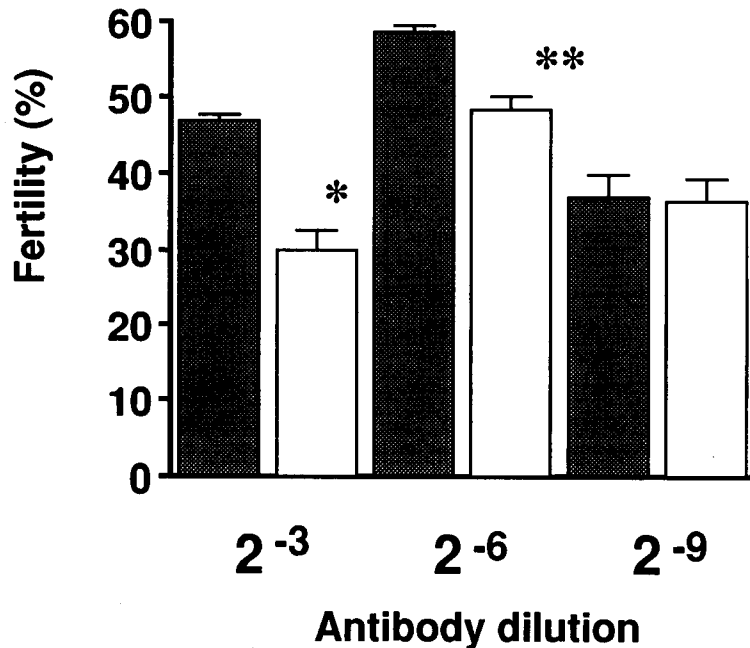


Fig. 1. Fertility of sperm coated with anti-sperm autoantibodies (open bar) and non-specific antibodies (shaded bar). \*\*,  $P < 0.001$ ; \*,  $P < 0.01$

Results

Fertility of sperm treated with autoantibodies at  $2^{-3}$  and  $2^{-6}$  dilutions averaged 30.0% and 48.2%, and that of sperm treated with non-specific antibodies was 46.8% and 58.5%, respectively (Fig. 1). At both dilutions, the differences of fertility

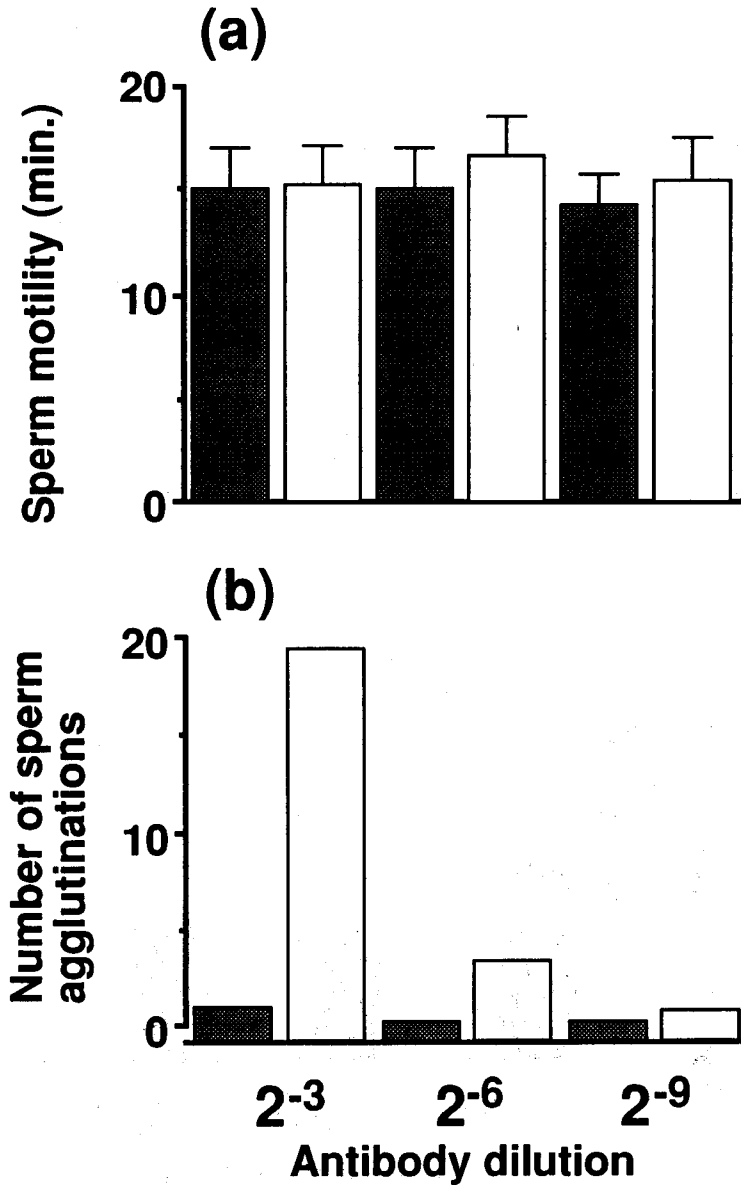


Fig. 2. Effects of coating sperm with anti-sperm autoantibodies (open bar) and non-specific antibodies (shaded bar) on sperm motility (a) and agglutination (b).

between the two antibody-treated groups of sperm were statistically significant ( $2^{-3}$ ,  $P < 0.001$ ;  $2^{-6}$ ,  $P < 0.01$ ). At  $2^{-9}$  dilution, however, no significant difference in fertility was observed between sperm treated with autoantibodies (36.2%) and that treated with non-specific antibodies (37%). Thus there was a tendency where the difference in fertility of sperm between the experimental and the control groups became larger in association with an increase in the concentration of antibodies. Sperm showed movements of a normal pattern and its motility lasted for more than 10 min in all groups (Fig. 2a), resulting in successful fertilization which seemed to be completed within a few min. These results suggest that the reduction of fertility of sperm coated with autoantibodies was not attributable to a deficiency in sperm motility. At  $2^{-3}$  dilution of autoantibodies, a number of sperm formed some large agglutinations, and only about 20% of sperm were seen to move actively (Fig. 2b). These agglutinations gradually became conspicuous after the sperm started to move. However, with autoantibodies and non-specific antibodies at lower dilutions, only a few or no sperm agglutination was formed.

At  $2^{-3}$  and  $2^{-6}$  dilutions, the ratio of sperm bound to autoantibodies was 83.1% and 78.8%, and that of sperm bound to non-specific antibodies was 44.9% and 31.3% on the average, respectively (Fig. 3). There were significant differences between the two groups ( $2^{-3}$ ,  $P < 0.001$ ;  $2^{-6}$ ,  $P < 0.05$ ) in the ratio of sperm bound to antibodies.

At  $2^{-9}$  dilution, the ratio of sperm bound to autoantibodies and to non-specific antibodies was 65.6% and 35.3%, respectively, and the difference was not statistically significant. Many spermatozoa that revealed apparent immunostaining on their surface and did not suffer from agglutination, were observed in autoantibody-

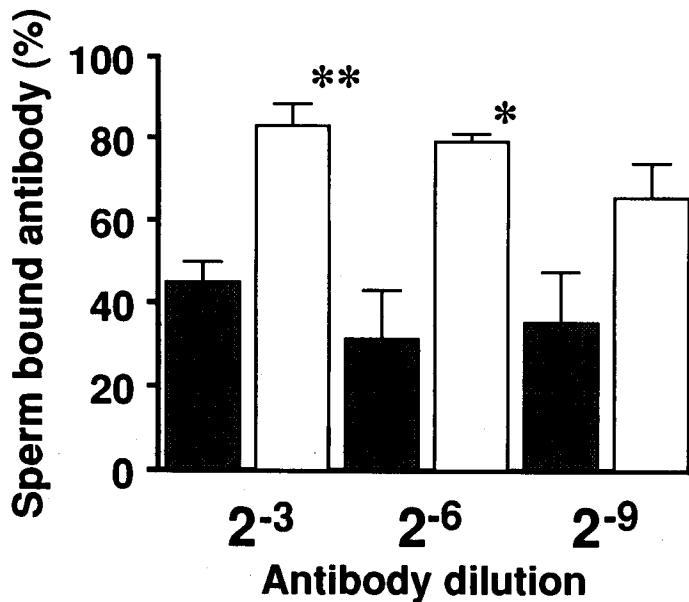


Fig. 3. A comparison of percentages of sperm bound to autoantibodies (open bar) and to non-specific antibodies (shaded bar) at three dilutions. \*\*,  $P < 0.001$ ; \*,  $P < 0.05$ .

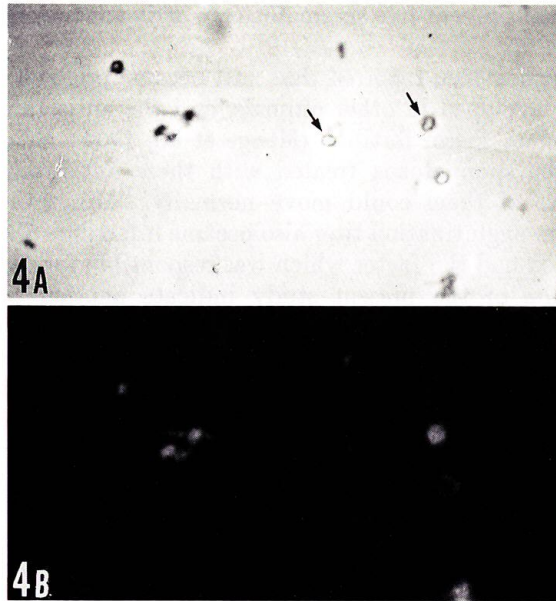


Fig. 4. Localization of autoantibodies bound to spermatozoa.

A. Phase contrast image of spermatozoa. ( $\times 570$ )

B. Immunofluorescent staining pattern of spermatozoa in the same field as A. Arrows indicate two spermatozoa, one of which does not bind the antibody. ( $\times 570$ )

treated groups at  $2^{-3}$  and  $2^{-6}$  dilutions (Fig. 4A, B).

### Discussion

In the present study, the fact that fertility of sperm coated with autoantibodies was apparently lowered in a concentration-dependent fashion as compared with that of sperm treated with non-specific antibodies, and that the coating of sperm with antibodies did not affect its motility, suggests that factors which are involved in fertilization may exist on the sperm plasma membrane in the Nile tilapia. The factors seem to be autoantigens. In the present study, different female fish were used to check the fertility at each of the three antibody dilutions, and it was possible that the quality of eggs used in the experiment at  $2^{-9}$  dilution was not good. This may explain the reason why the fertility at  $2^{-9}$  dilution was the lowest of all. In the case of  $2^{-9}$  dilution, there was no significant difference between the fertility of sperm reacting to autoantibodies and that of sperm reacting to non-specific antibodies. It would seem that this could be accounted for by the fact that there was no difference in the ratio of sperm bound to antibodies between the two groups of antibody treatments. Whereas autoantibodies and non-specific antibodies at  $2^{-6}$  and  $2^{-9}$  dilutions did not agglutinate spermatozoa, autoantibodies at  $2^{-3}$  dilution agglutinated them clearly. Although sperm agglutination may participate in the lowering of sperm fertility at that dilution of autoantibodies, it occurred fairly

gradually and did not prevent free spermatozoa to arrive at the micropyle to fertilize the egg.

In mammals, it has been reported that milt became infertile when it reacted to anti-milt antisera developed in other animals, and the antisera absorbed by testis material did not affect sperm fertility (Menge et al., 1962; Menge and Protzman, 1967). Some of the spermatozoa treated with these antisera were slightly agglutinated, but most of them could move normally. Milt treated with antisera showing a low sperm-agglutination titer also became infertile. These results suggest that the spermatozoa had the factor which was responsible for fertilization on their surface. The results of the present study indicate another possible factor for inducing sperm infertility in the fish immunized against testis material. It was shown in our recent study that a prominent reduction of sperm motility was caused in the tilapia immunized with allogeneic testis material emulsified with Freund's complete adjuvant (Mochida and Takahashi, unpublished). However, the reduction of sperm motility can not completely explain why the infertility was caused. Based on the assumption that sperm autoantigens are indispensably implicated in fertilization, one hypothesis is that the autoantibodies penetrate into efferent ducts and seminal lobules of the testes of the immunized fish and then attach to sperm surface autoantigens and block their function(s) responsible for fertilization. The autoantibodies may also activate the fish's own immune system at the same time.

In mammals, sperm surface autoantigens and isoantigens play important roles at various stages of fertilization from zona pellucida-sperm binding to embryonic development (Metz and Anika, 1970; Seki and Mettler, 1982; Naz et al., 1984; Primakoff et al., 1985, 1987). Also in the rainbow trout, *Oncorhynchus mykiss*, Trummel et al. (1992) reported that a positive correlation was established between fertility of sperm and the percentage of spermatozoa that have one antigenic epitope on their surface. This finding suggests that the antigenic epitope is involved in fertilization. Lou and Takahashi (1991) identified at least six kinds of sperm surface autoantigens in the Nile tilapia. It is likely that at least one of them may relate to fertilization, as indicated by the present study. To investigate functions of the other sperm surface autoantigens of the Nile tilapia, it is necessary to purify each of them and develop an antibody specific to each autoantigen. Monoclonal antibodies to the autoantigens of the Nile tilapia are now being prepared in our laboratory.

Sperm surface autoantigens must be the factors by which spermatozoa come to express their specific functions. Suzuki (1958) described that, in three species of bitterlings of the genus (*Acheilognathus*), spermatozoa were activated in the region around the micropyle area, and that a factor responsible for the activation existed in the micropyle area of the egg membrane. Yanagimachi (1957) reported similar results in Pacific herring, *Clupea pallasii*, and showed that, when the factor was inactivated, fertilization did not occur. If the sperm activator exists in the micropyle area of the egg membrane of the tilapia, the receptor of the activator in spermatozoa may be one of the autoantigens located on their surface.

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