

[Chem. Pharm. Bull.]
28(5)1497-1502(1980)

Studies on Sperm Capacitation. IX.¹⁾ Movement Characteristics of Spermatozoa in Relation to Capacitation²⁾

SHIGERU AONUMA, MASARU OKABE, MAKOTO KAWAGUCHI, and YUICHIRO KISHI

Faculty of Pharmaceutical Sciences, Osaka University³⁾

(Received October 26, 1979)

The changes of swimming patterns of spermatozoa accompanying capacitation were studied. The spermatozoa were seen to begin activated movement upon capacitation. The sperm activation was inhibited at low temperatures, in the absence of Ca^{2+} , upon addition of Zn^{2+} , and upon addition of Fr. I from guinea-pig spermatozoa which had decapacitation activity against rabbit spermatozoa. However, movement was not inhibited under the same conditions, when the spermatozoa had already acquired activated movement.

It was apparent that the activation of spermatozoa was inhibited only when the process of capacitation was suppressed. Thus it is suggested that sperm activation is an inherent property of sperm capacitation.

Keywords—capacitation; acrosome reaction; activation; *in vitro* fertilization; decapacitation factor; zinc; flagellum

It has been maintained that the mature spermatozoa stored in the cauda epididymidis might have the ability to achieve fertilization, in view of the fact that the spermatozoa began a very active movement when they were released in the female genital tract. However, Chang⁴⁾ and Austin⁵⁾ independently discovered that the spermatozoa need to reside for a time in the female reproductive tract in order to develop the capacity to fertilize the ova. Austin⁶⁾ termed this phenomenon 'capacitation'.

In a previous report,⁷⁾ we showed that a macromolecular substance (Fr. I) was released from the spermatozoa during incubation and that it had decapacitation activity. It was demonstrated that Fr. I also decreased the sperm motility and respiration.⁸⁾ Consequently, we suggested that capacitation is caused by the release of decapacitation factor(s) from the spermatozoa. Incidentally, Yanagimachi⁹⁾ has reported that the spermatozoa of golden hamster changed their swimming pattern when they completed capacitation ('activation' of the spermatozoa). Similar reports have appeared on the spermatozoa of guinea-pig¹⁰⁾ and dog.¹¹⁾

While we were making a basic study on *in vitro* fertilization of mouse ova, we observed that the spermatozoa went through a series of swimming patterns. An attempt was made to

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- 4) M.C. Chang, *Nature* (London), **168**, 697 (1951).
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- 10) a) R. Yanagimachi, *Anat. Rec.*, **174**, 9 (1972); b) C. Barros, M. Berrios, and E. Herrera, *J. Reprod. Fert.*, **34**, 547 (1973); c) R. Yanagimachi and N. Usui, *Exptl. Cell Res.*, **89**, 161 (1974).
- 11) C.A. Mahi and R. Yanagimachi, *J. Exp. Zool.*, **196**, 189 (1976).

study the relation between the changes of the swimming pattern and capacitation, while the spermatozoa were incubated under various conditions.

Experimental

The standard medium used throughout the experiments was modified Krebs-Ringer bicarbonate solution (m-KRB),¹²⁾ and it was used at pH 8.0 except when the effect of pH value was being examined.

Preparation of Sperm Suspension and Ova—The sperm suspension and ova were prepared by the method of Aonuma *et al.*¹³⁾ Epididymal spermatozoa were obtained from mice weighing 30–40 g. Cauda epididymides were chopped in 2 ml of m-KRB and 3 min later the upper part (1 ml) of the medium was sucked up with a Pasteur pipette as the sperm suspension ($1.0\text{--}1.5 \times 10^6$ spermatozoa/ml). The ova were obtained from immature ddy female mice, 4–5 weeks old, which were injected intraperitoneally with 5 i.u. PMSG (Teikoku Zoki) 48 hr before an *i.p.* injection of 5 i.u. hCG (Teikoku Zoki). The mice were sacrificed by cervical dislocation 14–16 hr after hCG injection. The oviducts were removed and placed in 5 ml of m-KRB in a watch glass. The ova were released from the oviduct ampullae, washed twice with medium and introduced into 0.4 ml of m-KRB.

In order to prevent change of pH, the sperm suspension and culture medium were placed under paraffin oil.

In Vitro Fertilization of Mouse Ova—*In vitro* fertilization of mouse ova was performed by the method of Aonuma *et al.*¹³⁾ Fifty μl aliquots of the sperm suspension were added to 0.4 ml of m-KRB containing 15–20 ova. The ova were incubated with spermatozoa at 37° under 5% CO₂ in air. The degree of sperm capacitation was estimated in terms of the proportion of penetrated ova having spermatozoa in the perivitelline space.

Temperature Effects—The sperm suspension (1.0 ml) obtained from a pair of cauda epididymides was divided into two parts. One part was incubated at 37° as a control. The other part was incubated at 20° or at 10° so that the effect of low temperatures could be examined.

pH Effects—The pH of the medium (pH 7.0, pH 8.0) was adjusted by the addition of sodium bicarbonate in the manner described previously.¹³⁾

Ion Effects—The effect of Ca²⁺ was observed by using m-KRB solution without Ca²⁺ (Ca²⁺-free m-KRB). The effect of Zn²⁺ was observed by using m-KRB containing 250 μM Zn²⁺ (Zn-KRB). The fertilizing ability of epididymal spermatozoa was maintained but latent during incubation in the presence of 250 μM Zn²⁺, as described previously.¹⁴⁾

Effect of Fr. I—Fr. I was obtained from guinea-pig spermatozoa by the method of our previous report.⁷⁾ Namely, during incubation of the spermatozoa in the medium, substances were released into the medium which were subsequently separated on Sephadex G-200 into two major fractions. The first peak (Fr. I) increased with sperm maturation and showed decapacitation activity. Fr. I (50 $\mu\text{g}/\text{ml}$) was added to the sperm suspension and incubated at 37° to examine its effect. The addition of Fr. I to the medium inhibited sperm penetration through the zona pellucida completely at 50 $\mu\text{g}/\text{ml}$.⁸⁾

Throughout the present experiments, the observations of the swimming patterns of spermatozoa were carried out at definite periods with a hemocytometer and phase contrast microscope ($\times 100$) fitted with a temperature-control apparatus. The percentage of spermatozoa exhibiting activation (see “Results”) was determined on the basis of the examination of approximately 100 spermatozoa.

Results

The observations of the swimming patterns of mouse epididymal spermatozoa in m-KRB (pH 8.0) at 37° showed that the spermatozoa moved rapidly and linearly immediately after the start of the incubation and gradually lost the linearity of movement, showing slow progress, with the head becoming twisted and flagellum being shaken. Forty minutes after the start of incubation, there was a noticeable change in the swimming behavior of the spermatozoa, characterized by vigorous and extreme flexing of the flagella. The movement no longer showed any direction, changing all the time in a sigmoid curve (activated spermatozoa, Fig. 1).

Sixty minutes later, the extent of spermatozoa activation reached a plateau level (Fig. 2).

The changes of the swimming pattern of spermatozoa in the medium of pH 8.0 were as described above. The activated spermatozoa at pH 7.0 showed the same appearance as at pH 8.0, though the changes were a little later (Fig. 2).

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13) S. Aonuma, M. Okabe, and Y. Kawai, *Yakugaku Zasshi*, **96**, 1307 (1976).

14) S. Aonuma, M. Okabe, and M. Kawaguchi, *J. Reprod. Fert.*, **53**, 179 (1978).

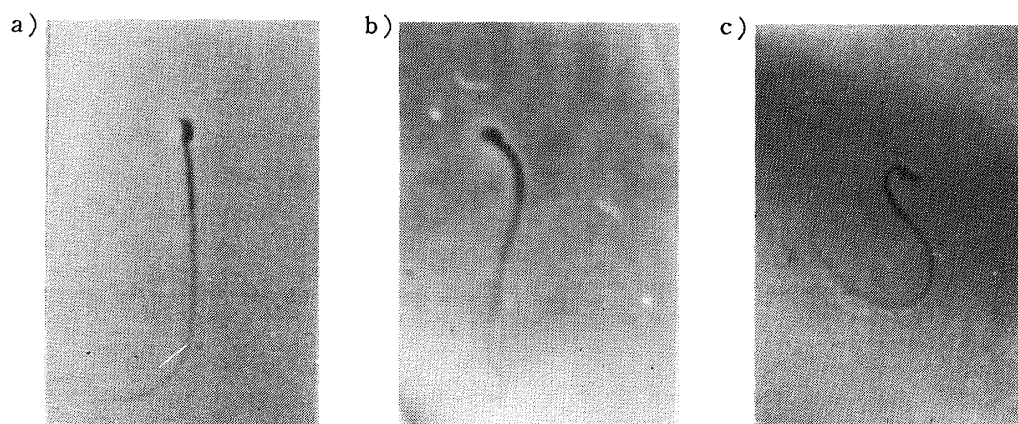


Fig. 1. Changes of Swimming Pattern of Mouse Spermatozoa during Incubation

A suspension (0.5 ml) of epididymal spermatozoa (about 1.0×10^6 spermatozoa/ml of m-KRB solution) was incubated at 37°.

- a) examined within 5 min of being suspended in m-KRB solution.
- b) examined after 20 min in m-KRB solution.
- c) examined after 60 min in m-KRB solution (an activated spermatozoon).

Activated spermatozoa hardly appeared when the epididymal spermatozoa were held at low temperatures (Fig. 3). On the other hand, the movement of spermatozoa after activation was not inhibited even at low temperatures (Fig. 4). Further, no marked decrease of the sperm motility was observed during incubation for 60 min at 20°.

It was found that the sperm penetration into the zona pellucida began 10–15 min after the activated spermatozoa appeared, as shown in Fig. 5.

The activated spermatozoa began to appear, following capacitation, about 40 min after a change of temperature from 20° to 37°. Subsequently, sperm penetration into the zona pellucida occurred (Fig. 5).

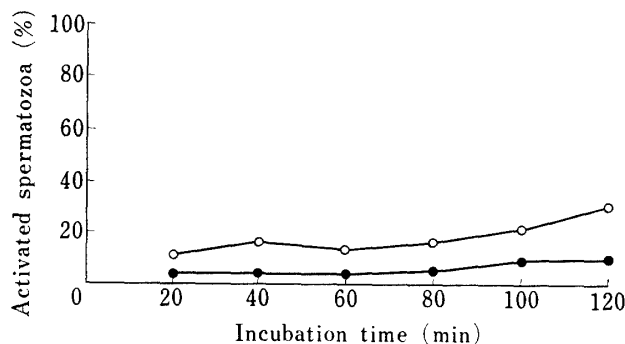


Fig. 3. Effect of Incubation Temperature on the Activation of Spermatozoa (I)

- , incubated in m-KRB solution at 20°.
- , incubated in m-KRB solution at 10°.

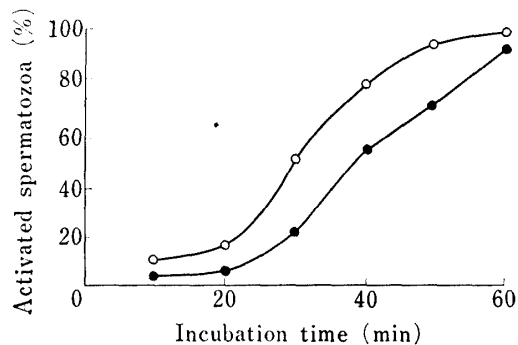


Fig. 2. Increase of Activated Spermatozoa during Incubation

- , incubated in m-KRB solution of pH 8.0 at 37°.
- , incubated in m-KRB solution of pH 7.0 at 37°.

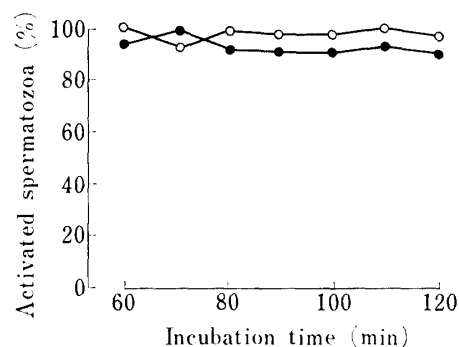


Fig. 4. Effect of Incubation Temperature on the Activation of Spermatozoa (II)

A suspension of epididymal spermatozoa was incubated for 60 min at 37°, and immediately transferred to 20° or 10°.

- , transferred from 37° to 20°.
- , transferred from 37° to 10°.

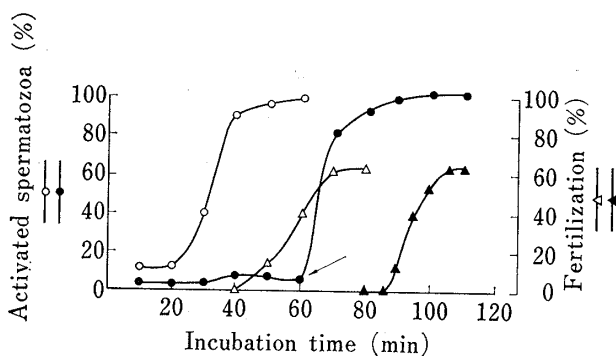


Fig. 5. Relationship between the Appearance of Activated Movement and Fertilizing Ability of Spermatozoa

—○—, —△—, incubated at 37°.
—●—, —▲—, incubated for 60 min at 20°, and immediately transferred to 37° (indicated by arrow).

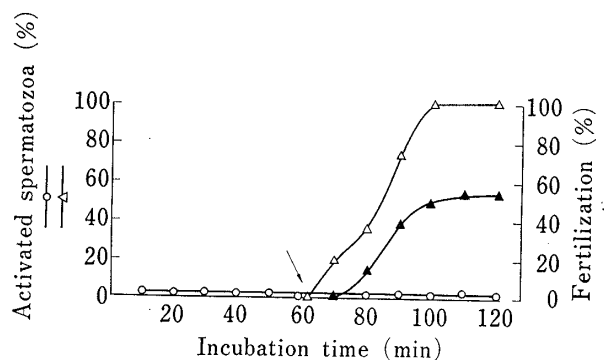


Fig. 6. Effect of Ca^{2+} on Activated Movement and Fertilizing Ability of Spermatozoa

—○—, incubated in Ca^{2+} -free m-KRB.
—△—, —▲—, incubated in Ca^{2+} -free m-KRB for 60 min, then Ca^{2+} added (1.71 mM, indicated by arrow).

In Ca^{2+} -free m-KRB, activated spermatozoa hardly appeared. However, movement began about 10 min after Ca^{2+} -free m-KRB was replaced with normal m-KRB, and then the spermatozoa were seen to penetrate the zona pellucida (Fig. 6).

On the other hand, activated spermatozoa were not seen in the presence of $250 \mu\text{M}$ Zn^{2+} , as shown in Fig. 7.

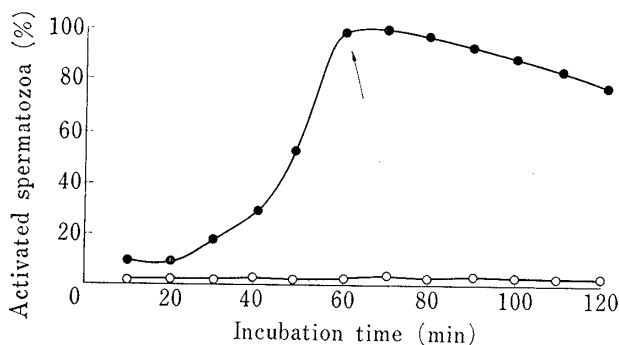


Fig. 7. Effect of Zn^{2+} on the Activation of Spermatozoa

—○—, incubated in Zn-KRB.
—●—, incubated for 60 min in m-KRB, then Zn^{2+} added ($250 \mu\text{M}$, indicated by arrow).

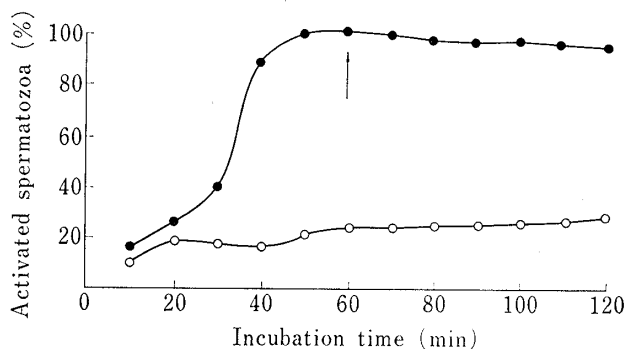


Fig. 8. Effect of Fr. I on the Activation of Spermatozoa

—○—, incubated in m-KRB containing Fr. I ($50 \mu\text{g/ml}$).
—●—, incubated in m-KRB for 60 min, then Fr. I added ($50 \mu\text{g/ml}$, indicated by arrow).

Even when Zn^{2+} was added to spermatozoa which had already begun the activation (spermatozoa 60 min after the start of incubation), activation continued without any change.

Furthermore, the activation of spermatozoa was inhibited in the presence of Fr. I ($50 \mu\text{g/ml}$), which had fertilization inhibitory activity towards mouse ova *in vitro* (Fig. 8).

On the other hand, the movement of spermatozoa after activation was not inhibited even when the spermatozoa were incubated in m-KRB containing Fr. I, as shown in Fig. 8.

Discussion

Yanagimachi^{9c)} reported for the first time the change of the swimming pattern of spermatozoa accompanying capacitation, using hamsters. Further, Yanagimachi¹⁵⁾ has reported that detergent induced accelerated activation of guinea-pig spermatozoa, which became

15) R. Yanagimachi, *Biol. Reprod.*, 13, 519 (1975).

capable of penetrating into the zona pellucida in a short time. However, some workers have reported that in the hamster, activation, capacitation and the acrosome reaction are independent phenomena.¹⁶⁾ Since it is difficult to observe the acrosome reaction in mice, as described by Bavister,^{16a)} the relation between the acrosome reaction and the activation of spermatozoa is not yet clear. Fraser¹⁷⁾ has reported a relationship between mouse sperm activation and fertilization. By *in vitro* fertilization of mouse ova, we observed the changes in the swimming pattern of spermatozoa and the ability to cause fertilization, in order to clarify the relation between sperm capacitation and the change of the swimming pattern. It was found that there was a parallel relationship between the appearance of spermatozoa with a particular swimming pattern (activated spermatozoa) and sperm penetration into the zona pellucida after capacitation.

In our previous paper,¹³⁾ it was suggested that the percentage of fertilization was very low at pH 7.0, not because sperm capacitation did not occur, but because sperm penetration into the zona pellucida was obstructed. In the present study, it was found that the activation of spermatozoa occurred even at pH 7.0. This supports the above-mentioned assumption.

Although the effect of ions on the spermatozoa is complicated because of environment and species differences, many workers have reported that Ca^{2+} is essential for fertilization.^{10c,18)} Incidentally, Miyamoto and Ishibashi¹⁹⁾ stated that even if mouse epididymal spermatozoa were capacitated by preincubation in a medium containing Ca^{2+} before mixing with ova, they were unable to penetrate the zona pellucida in a Ca^{2+} -free medium. It appears that Ca^{2+} in a chemically defined medium does not affect the process of sperm capacitation but does affect the penetration. Our findings on the swimming pattern of spermatozoa indicate that activated spermatozoa did not appear in Ca^{2+} -free m-KRB. This result is similar to that described by Fraser.¹⁷⁾ However, after the addition of Ca^{2+} , activated spermatozoa appeared immediately. Further, the period from Ca^{2+} addition to the beginning of sperm penetration into the zona pellucida was the same as in the case of capacitated spermatozoa. These findings suggest that capacitation was suppressed in the Ca^{2+} -free m-KRB, and at the same time, that some part of the capacitation process was occurring during incubation in the Ca^{2+} -free m-KRB, though it took 35 to 40 min in our *in vitro* fertilization of mouse ova.¹³⁾

Zn^{2+} is present in the seminal plasma in abundance, and we have reported that Zn^{2+} has capacitation inhibitory activity which is physiologically significant, since Zn^{2+} did not show any fertilization inhibitory activity on capacitated spermatozoa, whereas it showed fertilization inhibitory activity on the epididymal spermatozoa at a concentration of 250 μM .¹⁴⁾ As expected, it was found that the appearance of the activated spermatozoa was suppressed in the presence of 250 μM Zn^{2+} . Further, Zn^{2+} hardly affected activated spermatozoa.

It is apparent that the activation of spermatozoa is inhibited only when the process of capacitation is suppressed.

In our previous paper,⁷⁾ it was shown that the macromolecular substance Fr. I was released from the spermatozoa during incubation. Fr. I from guinea-pig spermatozoa which had decapacitation activity against rabbit spermatozoa showed fertilization inhibitory activity towards mouse ova *in vitro*.⁸⁾ As one mechanism of capacitation, Aonuma *et al.*⁷⁾ demonstrated that the decapacitation factor(s) was combined with spermatozoa in the epididymidis and that capacitation might occur by removal of the decapacitation factor(s) from the spermatozoa. As shown in Fig. 8, the activation of spermatozoa was inhibited in the presence of Fr. I. Con-

16) a) B.D. Bavister, *J. Reprod. Fert.*, **35**, 161 (1973); b) C.A. Mahi and R. Yanagimachi, *J. Reprod. Fert.*, **35**, 55 (1973); c) C.A. Mahi and R. Yanagimachi, *Biol. Reprod.*, **9**, 73 (1973); d) C. Barros and M. Berrios, *J. Exp. Zool.*, **201**, 65 (1977).

17) L.R. Fraser, *J. Exp. Zool.*, **202**, 439 (1977).

18) a) T. Iwamatsu and M.C. Chang, *J. Reprod. Fert.*, **26**, 197 (1971); b) R. Yanagimachi, *Anat. Rec.*, **172**, 430 (1972); c) B.K. Davis, D.M. Hunt, and M.C. Chang, *Proc. Soc. Exp. Biol. Med.*, **147**, 479 (1974).

19) H. Miyamoto and T. Ishibashi, *J. Reprod. Fert.*, **45**, 523 (1975).

sequently, taking into account the swimming patterns of spermatozoa, it seems that Fr. I and Zn^{2+} play important roles in the capacitation process of spermatozoa.

It is not clear at present whether sperm activation is a universal phenomenon. The changes of swimming patterns of spermatozoa in *in vitro* observations such as the present ones do not appear to be entirely unphysiological. Spermatozoa within the oviducts of naturally mated females display typically activated movement at the site and time of fertilization.^{9c)}

As for sperm transport in the female genital passage, it is necessary to consider sperm transport in relation to the movement of the female genital passage.²⁰⁾ We put forward a hypothesis on sperm transport in relation to the swimming patterns of spermatozoa at the same time as Lewis and Bierwolf's report,²⁰⁾ which described the period needed for mouse spermatozoa to reach the upper part of the uterine tube. Assuming that after ejaculation, the spermatozoa progress straight through the vagina and uterus, and enter the oviduct, it is presumed that the spermatozoa will complete capacitation before entry into the oviduct, and that they will begin movement at a fixed point. Since fertilization occurs in the oviduct ampullae, the activation of spermatozoa in the oviduct must serve to increase the spermatozoa concentration at the region of fertilization.^{12,13,18a,21)} Furthermore, large-amplitude flagella undulations are assumed to provide motive power for the spermatozoa²²⁾ to penetrate the cumulus oophorus and zona pellucida for the purpose of fertilization. In other words, flagellation is assumed to have significant physiological functions.

20) A. Iritani, *Taisha*, **16**, 299 (1979).

21) a) Y. Toyoda, M. Yokoyama, and T. Hoshi, *Jap. J. Anim. Reprod.*, **16**, 152 (1971); b) Y. Tsunoda and M.C. Chang, *J. Reprod. Fert.*, **44**, 139 (1975); c) D.P. Wolf and M. Inoue, *J. Exp. Zool.*, **196**, 27 (1976).

22) J. Lighthill, *S.I.A.M. Rev.*, **18**, 161 (1976).