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Fertilizing Capacity of Dog Salmon Spermatozoa in Ringer's Solution, with Special Reference to the Effect of Dilution

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Abstract Spermatozoa of the dog salmon, *Oncorhynchus keta*, when suspended in Ringer's solution lost their fertilizing capacity within 20 minutes. However, such a suspension restored this capacity when it was diluted with fresh Ringer's solution. For the fertilization of dog salmon eggs with spermatozoa suspended in Ringer's solution, it is therefore desirable that the sperm suspension in which the eggs are immersed be diluted with fresh Ringer's solution prior to removing the eggs to tap water for the initiation of embryonic development. By this procedure, spermatozoa suspended in Ringer's solution still fertilized some 10% of eggs after 120 minutes. The mechanism underlying the effect of dilution as increasing the fertilizing capacity of sperm suspension is discussed in relation to the inhibition of sperm movement.

The inactivation of the genetic materials of the sperm nucleus is one of the methods for understanding the role of spermatozoa in fish development. In order to inactivate the genetic materials, various agents have been used. For example, Oppermann (1913 a and b) carried out the inactivation by the irradiation of trout semen with radium. Several chemicals are also useful for this purpose (Uwa, 1965; Jones et al., 1975). At the time of use, solutions of these chemicals, dissolved at very low concentrations, are usually mixed with semen. As is well known, spermatozoa of freshwater fishes suspended in water die within a short time. If the suspension is made with isotonic salt solution, however, they survive for a certain time (Schlenk and Kahmann, 1938; Ellis and Jones, 1939). For this reason, the chemicals used for the inactivation of sperm nuclei should be dissolved in Ringer's solution (Uwa, 1965). On the fertilizing capacity of salmonid spermatozoa suspended in Ringer's solution, there is a conspicuous difference between the experimental results of Kusa (1950) and Ginsburg (1963). The former author described a gradual reduction of the fertilizing capacity of dog salmon spermatozoa in Ringer's solution. According to his data, the fertilizing capacity of a suspension is completely lost after 90 minutes. On the other hand, Ginsburg reported that lake trout spermatozoa suspended in Ringer's solution rapidly lost their fertilizing capacity within 10 minutes but the capacity was gradually restored. To perform the inactivation of sperm nuclei with chemicals, an exact knowledge of the fertile life of spermatozoa in Ringer's solution is required. Therefore, in the present report, the author reexamined the fertilizing capacity of dog salmon spermatozoa suspended in Ringer's solution. The effect of diluting the suspension with fresh Ringer's solution will be also described.

Material and method

Materials used in the present study were the dog salmon, *Oncorhynchus keta* (Walbaum), captured at the Chitose Salmon Hatchery, Hokkaido. The ripe eggs were obtained by dissecting the belly of mature females. By manually exerting pressure upon both sides of the belly of mature males, the ejaculation of semen was induced. Care was taken in order to avoid the contamination of the ejaculated semen with blood and excrement. Those gametes which showed over 90% cleavage after fertilization by the dry method were used for the experiments. The Ringer's solution had the following constitution: M/7 NaCl 100 parts + M/7 KCl 2.8 parts + M/10 $CaCl_2$ 3.4 parts (pH 7.3). Except for the cultivation of the inseminated eggs in tap water, the experiments were carried out at 4°C.

Results

Concentration of sperm suspension effective for inducing a high percentage of fertilization

Before studying the fertile life of spermatozoa in Ringer's solution, it is desirable to know what dilutions of the semen with Ringer's solution are required to obtain a high percentage of fertilization. The concentrations of spermatozoa in the semen used varied with different males and were 189.2, 213.0 and 265.6×10^8 spermatozoa/ml semen respectively. One ml of the semen obtained from a single male was mixed with certain amounts (from 1 to 500 ml) of Ringer's solution in glass tubes and the mixtures were vigorously shaken for 2 minutes. About 30 ml of these sperm suspensions were immediately poured into clean petri-dishes, each of them containing 100 ripe eggs derived from a single female. Two minutes later, about 70 ml of fresh Ringer's solution were added to these dishes. After 30 minutes, the eggs thus treated were transferred into tap water and allowed to develop for 18 hours at 10°C. The eggs were then fixed in Bouin's fluid and their cleavage rates were examined under the dissecting microscope. At the time of fixation, the fertilized eggs had reached 8 cell stage.

A typical result of these experiments is summarized in Fig. 1. As will be seen, the cleavage rates were nearly constant over the range of dilutions examined. According to the earlier reports (Smirnov, 1963; T.S. Yamamoto, 1974), the variations in the concentration of spermatozoa in semen from different individuals are relatively small and the concentrations are from 56.0 to 324.0×10^8 spermatozoa/ml semen. Therefore it may be said that, at these levels of dilution, the variations in the concentration of spermatozoa in semen owing to the individual differences of the male are negligible for the purpose of the present study. On the basis of these considerations, the sperm suspensions used in the following experiments were always made by mixing 1 ml of semen with 300 ml of Ringer's solution.

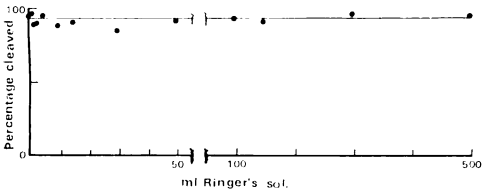

Fig. 1. Fertilizing capacity of various concentrations of spermatozoa suspended in Ringer's solution. The sperm suspension was made by mixing 1 ml of semen with various amounts of Ringer's solution indicated in abscissa. Concentration of spermatozoa in the semen; 265.6×10^8/ml.

Fertile life of spermatozoa in Ringer's solution

About 30 ml of the sperm suspension were poured at various intervals into clean petri-dishes containing 100 ripe eggs. Two minutes later, the eggs were directly transferred into tap water and fixed in Bouin's fluid after 17 hours.

The results indicate that spermatozoa rapidly lost their fertilizing capacity in Ringer's solution (Fig. 2). No eggs were fertilized by 20 minute-old sperm suspension. However, the sperm suspension slightly recovered its fertilizing capacity after a further 30~80 minutes and 7% of eggs showed cleavage after insemination with 80 minute-old sperm suspension.

It should be noted in Fig. 2 that the cleavage rate of the eggs inseminated by 2 minute-old suspension was about 1/2.5 of the rate of those inseminated by the dry method. Since in the first experiment the cleavage rate of the eggs inseminated with 2 minute-old sperm suspension was nearly the same as for those inseminated by the dry method, the above mentioned reduction of the cleavage rate seemed a very peculiar fact. Then the procedures used in these two kinds of experiments were compared. In the first experiment, the ripe eggs were inseminated with the sperm suspension; 2 minutes later, the sperm suspension in which the eggs were immersed was

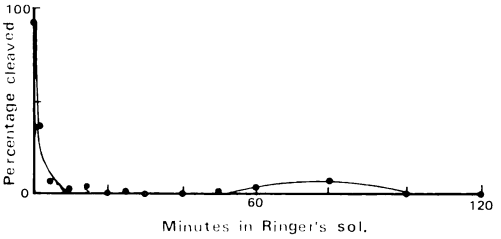

Fig. 2. Effect of Ringer's solution upon fertilizing capacity of spermatozoa. The sperm suspension was made by mixing 1 ml of semen with 300 ml of Ringer's solution and was allowed to stand for various periods of time as indicated in abscissa. Eggs immersed in this suspension were directly transferred into tap water. The value shown at 0 min is that obtained by the dry method.

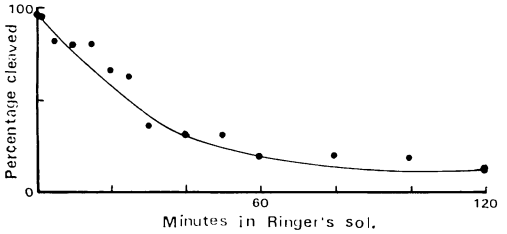

Fig. 3. Effect of Ringer's solution upon fertilizing capacity of spermatozoa. The sperm suspension in which the eggs were immersed was made in the same manner as indicated in Fig. 2 but was diluted with fresh Ringer's solution prior to being replaced by tap water. The value shown at 0 min is that obtained by the dry method.

diluted with fresh Ringer's solution, then was replaced with tap water. On the other hand, in the experiment described in this section, although the ripe eggs were inseminated with the sperm suspension in the same manner, the sperm suspension in which the eggs were immersed was replaced with tap water without dilution by fresh Ringer's solution. Therefore the only difference between these two procedures was whether the sperm suspension in which the eggs were immersed was diluted with fresh Ringer's solution prior to being replaced with tap water or not. Perhaps such a difference in the experimental procedure could cause the difference in results. Therefore, in order to test this possibility, 70 ml of fresh Ringer's solution was added to the petri-dish prior to the replacement of the sperm suspension with tap water and the dish was allowed to stand for 30 minutes.

The results were that the cleavage rate of the eggs inseminated with 2 minute-old suspension was nearly the same as for those fertilized by the dry method. Furthermore it was revealed that the fertilizing capacity of the sperm suspension reduced gradually with the lapse of time but about 12% of the eggs were still fertilized even by 120 minute-old suspension (Fig. 3).

Discussion

When the semen of dog salmon was diluted with Ringer's solution, the fertilizing capacity of the sperm suspension was considerably reduced within 2 minutes and was lost after about 20-minutes. The capacity was, however, slightly restored by a further 30～80 minutes. These results nearly coincide with those obtained by Ginsburg (1963) in lake trout spermatozoa. If the experimental procedure was altered, however, the results varied. That is; when the sperm suspension containing the eggs was diluted with fresh Ringer's solution prior to being replaced with tap water, the fall in the fertilizing capacity of the suspension was gradual, so that after 120 minutes the semen diluted with Ringer's solution fertilized some 10% of the eggs. These results are similar to those obtained by Kusa (1950). It appeared therefore that the variation in the time course of the reduction of fertilizing capacity found between the results of Kusa (1950) and Ginsburg (1963) does not originate from the difference of the species but is due to the variety of the procedure for the insemination and activation of the ripe eggs.

Ginsburg (1963) observed that spermatozoa show an active movement at the time of the dilution of semen with Ringer's solution but the bulk of them become motionless within 1 minute. This fact suggests that the reduction of fertilizing capacity of the sperm suspension has a relation to the rapid decline of the motility of spermatozoa in Ringer's solution. In fact, Kanoh (1957) has stated that the active movement of spermatozoa is indispensable for its passing through the micropylar canal in the process of fertilization of the dog salmon egg.

As mentioned already, if the sperm suspension containing the eggs is diluted with fresh Ringer's solution prior to being replaced with tap water, the cleavage rate of the eggs increased considerably. Therefore it can be said that the rapid loss of fertilizing capacity of the sperm suspension is a reversible one. Now, a question arises. Why does the sperm suspension rapidly lose its fertilizing capacity but recovers that capacity by dilution with fresh Ringer's solution? There are two possibilities. The one is that the bulk of spermatozoa in the semen exhaust their energy during the active movement occurring at the time of the first contact with Ringer's solution. However, some spermatozoa remain motionless at this time and the initiation of movement is retarded until the dilution of the suspension with fresh Ringer's solution. Since salmon eggs remain in the fertilizable state for a certain time in Ringer's solution (K. Yamamoto, 1951),

additional fertilization of eggs which were not fertilized in the suspension is accomplished by these spermatozoa. The additional fertilization results in the increase of the cleavage rate. If this holds true, the reason why some spermatozoa persist in the motionless state at the first contact with Ringer's solution but become active by the dilution of the suspension remains unknown.

The other possibility is that the same spermatozoon immobilized after the first contact with Ringer's solution recovers its activity and penetrates into an egg previously not fertilized, the cleavage rate thus being increased. Based on analytical data of the trout seminal plasma, Schlenk and Kahmann (1938) supposed that the lack of motility of spermatozoa in the seminal plasma is due to the concentration equilibrium of K ions (Konzentrationsgleichgewichts von Kaliumionen) between the sperm tail and the environmental solution. If the equilibrium does not exist, spermatozoa seem to show an active movement. Since the content of K ions in the Ringer's solution used in the present study is apparently lower than that of the seminal plasma reported by these authors, the movement observed at the time of dilution of semen with Ringer's solution might be caused by the decrease of K-content in the sperm tail. Probably the equilibrium of K ions between the sperm tail and Ringer's solution is soon attained with a minimum loss of K ions from the tail, by which the spermatozoa might soon become immobile in Ringer's solution. However these motionless spermatozoa recovered their activity and again showed a sliding movement after dilution of the suspension with fresh Ringer's solution. Since the equilibrium of K ions would have been already attained between the tail and Ringer's solution, the reappearance of the sliding movement of the spermatozoa may not be accompanied by the loss of K ions from the tail.

According to Rothschild (1958), spermatozoa of the salmon cannot be induced to move by increasing the oxygen tension. Therefore the dilution effect observed in the present study is not due to increased oxygen tension. A substance inhibiting the sperm movement, androgamone I, has been obtained by methanol extraction of salmon and trout spermatozoa (Runnström et al., 1944; Hartmann et al., 1947). Rothschild (1958) has stated that, even if salmon or trout spermatozoa contain an organic substance which inhibits sperm movement, the substance does not occur normally in the seminal plasma. If the supposition that the same spermatozoon recovers its fertilizing capacity after dilution of the suspension is correct, then the results of the present study seem to indicate the occurrence of some substance inhibitory to sperm movement, other than K ions, in dog salmon spermatozoa. The substance may prevent the penetration of the spermatozoon into the egg by immobilizing the spermatozoa at a very low concentration and is only effective when it is released from spermatozoa to the environmental solution.

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Literature cited

Ellis, W. G. and J. W. Jones. 1939. The activity of the spermatozoa of *Salmo salar* in relation to osmotic pressure. J. Exp. Biol., 16: 530～534.

Ginsburg, A. S. 1963. Sperm-egg association and its relationship to the activation of the egg in salmonid fishes. J. Embryol. Exp. Morph., 11: 13～33.

Ginsburg, A. S. 1972. Fertilization in fishes and the problem of polyspermy. Translated into English. Israel Program for Scientific Translations, Jerusalem.

Hartman, M., F. G. Medem, R. Kuhn, and H. J. Bielio. 1947. Untersuchungen über die Befruchtungsstoffe der Regenbogenforelle. Z. Naturforsch., 2: 330～349.

Jones, P., H. Jackson, and M. H. S. Whiting. 1975. Parthenogenetic development after chemical treatment of *Xenopus laevis* spermatozoa. J. Exp. Zool., 192: 73～82.

Kanoh, Y. 1957. Notes on the mechanism of monospermic fertilization in fish eggs. J. Fac. Sci. Hokkaido Univ. Ser. VI, Zool., 13: 394～398.

Kusa, M. 1950. Physiological analysis of fertilization in the egg of salmon, *Oncorhynchus keta*. I. Why are the eggs not fertilized in isotonic Ringer solution? Annot. Zool. Japon., 24: 22～28.

Oppermann, K. 1931a. Die Entwicklung von Forelleneiern nach Befruchtung mit radiumbestrahlten Samenfäden. Arch. Mikr. Anat., Abt. II, 83:

141～189.
Oppermann, K. 1913b. Die Entwicklung von Forelleneiern nach Befruchtung mit radiumbestrahlten Samenfäden. II. Teil. Das Verhalten des Radiumchromatins während der ersten Teilungsstadien. Arch. Mikr. Anat., Abt. II, 83: 307～323.
Rothschild, L. 1958. Fertilization in fish and lampreys. Biol. Rev., 33: 372～392.
Runnström, J., S. Lindvall, and A. Tiselius. 1944. Gamones from the sperm of sea urchin and salmon. Nature, 153: 285～286.
Schlenk, W. and H. Kahmann. 1938. Die chemische Zusammensetzung des Spermaliquors und ihre physiologische Bedeutung. Biochem. Zeitsch., 295: 283～301.
Smirnov, A. I. 1963. Production of spermatozoa in Pacific salmon of genus *Oncorhynchus*. Ikhtiologii, 3: 84～98. Indirectly cited from Ginsburg (1972).
Uwa, H. 1965. Gynogenetic haploid embryos of the medaka (*Oryzias latipes*). Embryologia, 9: 40～48.
Yamamoto, K. 1951. Activation of the egg of the dog-salmon by water and the associated phenomena. J. Fac. Sci. Hokkaido Univ. Ser. VI, Zool., 10: 303～318.
Yamamoto, T. S. 1974. DNA and RNA content of the dog salmon egg during early embryonic development. J. Fac. Sci. Hokkaido Univ. Ser. VI, Zool., 19: 489～502.

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リンゲル液中におけるサケ精子の受精力と希釈の影響

山本　正

リンゲル液に懸濁したサケ *Oncorhynchus keta* の精子は 20 分以内に受精力を失う．しかし，このような懸濁液はリンゲル液で希釈すると受精力を回復する．従って，リンゲル液に懸濁させた精子でサケ卵を受精させるには発生開始の為に卵を水に移す前に，卵を浸していた精子懸濁液をリンゲル液で希釈することが望ましい．この方法によると，リンゲル液に懸濁した精子は 120 分後にもなお 10% 前後の卵を受精させる．精子懸濁液の受精力増強を起させる希釈の影響機構を精子の運動抑制との関連で論じた．

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