Effects of HeLa and BHK-21 cells on the survival of fowl, bull, ram and boar spermatozoa *in vitro*

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Summary. HeLa and BHK-21 cells, which are not related immunologically with fowl cells, were cultured and the effects of these cells on the survival and fertilizing ability of fowl spermatozoa were investigated.

When spermatozoa were incubated at 41°C with these cells, their motility was maintained for 5 to 6 days. Fertilizing ability was also highly retained after incubation at 41°C for 2 days.

The use of cultured cells could also contribute to the maintenance of the motility of bull and ram spermatozoa, but this system could not be applied to boar spermatozoa.

Introduction

Temperature has long been known to exert a strong influence on the motility, survival and fertilizing ability of spermatozoa (Mann, 1964). Rabbit spermatozoa, soon lose

their motility at 45°C, although they are capable of surviving for several hours *in vitro* at 40°C (Walton, 1930). In the domestic fowl, fertilizing ability of spermatozoa stored in Ringer's solution at 41°C, the normal body temperature, is also lost within a few hours (Schindler, Weinstein, Moses & Gabriel, 1955).

Recently, however, fowl spermatozoa incubated at 41°C with the primary cultured cells from the fowl oviduct, kidney and skeletal muscle of a 9-day chick embryo were able to maintain their motility for 5-7 days and fertilizing ability for 2-4 days (Ashizawa, Nishiyama & Nagae, 1976; Ashizawa & Nishiyama, 1977; Fujihara & Howarth, 1980). These results indicate that the storage of fowl spermatozoa with cultured cells is a very effective means for prolonging sperm survival, and suggest that this effect may be observed with many other sources of cultured cells.

In the present study, we investigated the effectiveness of different cultured cells such as HeLa (Gey, Coffman & Kubicek, 1952) and BHK-21 (MacPherson & Stoker, 1962) cells, which are not related immunologically with fowl cells. We also studied whether the prolongation of sperm survival by this system could be applied to some mammalian spermatozoa.

Materials and Methods

Animals

Normal mature animals, White Leghorn roosters and pullets

(Shaver strain) obtained from Koyu Poultry Farm, Miyazaki, Japan, Holstein-Friesian bull and Corriedale ram obtained from Sumiyoshi Farm in Miyazaki University, Miyazaki, Japan, and Hampshire boar obtained from Miyazaki Prefectural Livestock Experiment Station, Miyazaki, Japan, were used throughout the study.

Survival and fertilizing ability of fowl spermatozoa in the presence of HeLa and BHK-21 cells

Monolayer cultures of HeLa and BHK-21 cells were obtained in using Eagle's MEM supplemented with 10 % newborn calf serum (Commonwealth Serum Laboratories) at 41°C in 5 % CO_2 + 95 % air for 3 days until they reached confluence in 2.5 cm Petri dishes. Kanamycin sulphate (60 µg/ml) was routinely added to the medium. At the same time, the cells originating from epithelia of the shell gland of the pullet oviduct were cultured under the same conditions (control 1). Cell preparations and cultures were carried out according to the method described by Ashizawa et al. (1976). On the 3rd day of culture, the culture medium (1 ml) was changed and fowl spermatozoa were added to reach a sperm concentration of 2.0×10^8 / ml. Spermatozoa added were collected as aseptically as possible from several roosters by the method of Fujihara, Tanaka & Nishiyama (1973) and washed once with culture medium by centrifuging the suspension at 500 g for 10 min. The spermatozoa and cells were incubated

as described above for the culture of cells alone. Spermatozoa incubated in cell-free culture medium were used as control 2. After addition of semen, sperm motility was assessed at room temperature (20-25°C) every day during the incubation period at 41°C. The evaluation of motility was carried out by observing spermatozoa from several areas (usually 4 or 5) of the Petri dish directly from above using an inverted microscope, and their motility was scored on a 0 to 5 scale (Wheeler & Andrews, 1943). The mean percentage of morphologically abnormal spermatozoa was determined on the 2nd and 4th day of incubation (see Ashizawa et al., 1976). Fertilizing ability was examined using the fowl spermatozoa incubated with cultured cells or in the cell-free medium for 2 days. Approximately 1.2 X 10^8 spermatozoa in about 0.6 ml culture medium were inseminated into the shell gland of pullet's oviduct by the method of Bobr, Lake, Lorenz, Ogasawara & Krzanowska (1965). From 2 days after insemination, any eggs laid were collected, incubated and examined on the 4th day of incubation for fertility. The duration of fertility was also determined.

Survival of bull, ram and boar spermatozoa in the presence of HeLa and BHK-21 cells

The cells to be cultured were HeLa and BHK-21 cells. The method for culturing the cells and the medium used were as described above. Kanamycin sulphate (60 µg/ml), penicillin G potassium (100 units/ml), streptomycin sulphate

(100 μg/ml) and tetracycline hydrochloride (50 μg/ml) were routinely added to the medium. Mammalian spermatozoa (bull, ram and boar) and fowl spermatozoa which were collected as aseptically as possible were added into the Petri dishes to reach a sperm concentration of 2.0×10^8 / ml. The procedures for collecting semen were as follows: artificial vagina (bull), electrical stimulation (ram) and gloved-hand technique (boar). Boar semen used consisted of sperm-rich fractions of the ejaculates. Collection of fowl semen was as described above. The spermatozoa and HeLa cells or BHK-21 cells were incubated at 41°C (close to the fowl's body temperature) or 38°C (close to the bull, ram and boar's body temperature) in 5 % CO_2 + 95 % air. Spermatozoa incubated in cell-free medium were used as controls. Sperm motility was assessed at room temperature (20-25°C) every 3 hours by the method described above for the evaluation of fowl sperm motility, and duration of the maintenance of motility of spermatozoa was determined. Observation of boar sperm motility was carried out after shaking of the Petri dish because spermatozoa showed anabiosis.

Results and Discussion

Effects of incubation with HeLa and BHK-21 cells on the maintenance of motility, incidence of abnormality and fertilizing ability of fowl spermatozoa

The changes of sperm motility in the various incubation experiments are shown in Table 1. Fowl spermatozoa in

the cell-free medium (control 2) lost their motility rapidly, while those incubated with cells of any source maintained their motility for 5 to 6 days and there were almost no differences in the effects of three types of cultured cells. The numbers of morphologically abnormal spermatozoa were also lower in the incubation experiment with cultured cells than in control 2 and the differences between them were highly significant ($P<0.01$) (Table 2).

The fowl spermatozoa incubated for 2 days at 41°C in cell-free medium had no fertilizing ability. In contrast, spermatozoa incubated with the cultured cells were highly fertile as judged by the number of fertile eggs laid during the 1st and 2nd week after insemination (Table 3). Not only fowl oviducal cells but also HeLa and BHK-21 cells exerted similar favourable effects on the maintenance of the fertilizing ability of spermatozoa and there were no differences between them in the duration of fertility ($P>0.5$). From these results, it is considered that the effectiveness of cultured cells for prolonging the survival of fowl spermatozoa may be found in many other sources of cultured cells.

Effects of incubation with HeLa and BHK-21 cells on the maintenance of motility of bull, ram and boar spermatozoa

The results are shown in Table 4 and 5. Bull and ram spermatozoa as well as fowl spermatozoa incubated with cultured cells were also able to prolong their motility about 2-3 times as compared with those incubated in

cell-free medium. These results show that all kinds of cultured cells tested are effective for prolonging the survival of the spermatozoa of several mammalian species. However, the use of cultured cells could not contribute to the maintenance of the motility of boar spermatozoa for unknown reasons. When the incubation temperature of spermatozoa was compared, it was found that the survival period in mammalian spermatozoa was slightly longer at 38°C than at 41°C, but in the fowl spermatozoa, there was no difference in survival ability between the two temperatures.

In general, it is considered that the nutrients required for the maintenance of cells or spermatozoa during the incubation experiments are limited in a small Petri dish. Thus, incubation of spermatozoa alone seems to be more favourable for the prolongation of survival than that with a combination of spermatozoa and cultured cells. However, the results obtained from the present study were somehow unexpected. Indeed, it would be of interest to determine the reasons why spermatozoa incubated with cultured cells are able to survive for a long period of time. There is a possibility that a substance or substances which are supplied by the cultured cells and reach the spermatozoa by diffusion may be effective for prolonging the survival of spermatozoa (Ashizawa & Nishiyama, 1978; Fujihara & Howarth, 1980). However, the nature of the "prolonged survival factor(s)" is not yet understood.

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Table 1. Motility score (at room temperature, 20-25°C) of fowl spermatozoa incubated with cultured cells at 41°C in 5 % CO_2 + 95 % air

Cells in incubation medium	Days after addition of spermatozoa							
	0	1	2	3	4	5	6	7
HeLa cells	5.0	4.6	3.6	2.8	1.6	1.0	0.4	0.0
BHK-21 cells	5.0	4.4	3.6	2.8	1.4	0.4	0.0	0.0
Epithelial cells of fowl oviduct (control 1)	5.0	4.4	3.6	3.0	1.4	0.6	0.0	0.0
None (control 2)	5.0	3.4	0.4	0.0	0.0	0.0	0.0	0.0

Each figure represents an average of 5 trials.

Table 2. The mean ± S.E.M. (of 5 trials) percentage of morphological abnormal fowl spermatozoa after incubation in various media for 2 or 4 days

Cells in incubation medium	Incubation for 2 days	Incubation for 4 days
HeLa cells	13.4 ± 1.2 [a]	30.2 ± 1.1 [c]
BHK-21 cells	13.5 ± 1.6 [a]	35.8 ± 1.3 [c]
Epithelial cells of fowl oviduct (control 1)	12.6 ± 0.6 [a]	33.7 ± 1.1 [c]
None (control 2)	88.5 ± 1.5 [b]	90.6 ± 2.2 [d]

Within columns, values with different superscripts differ significantly ($P < 0.01$) from each other.

Table 3. Fertilizing ability of fowl spermatozoa incubated with cultured cells for 2 days at 41°C in 5 % CO_2 + 95 % air

Cells in incubation medium	No. of hens inseminated	Fertilized eggs (%)		Mean duration of fertility (days)
		1st week	2nd week	
HeLa cells	4	95.5 (21/22)*	76.0 (19/25)	13.8 [a]
BHK-21 cells	4	91.3 (21/23)	87.3 (21/24)	15.0 [a]
Epithelial cells of fowl oviduct (control 1)	4	95.8 (23/24)	82.6 (19/23)	13.3 [a]
None (control 2)	4	0.0 (0/22)	0.0 (0/24)	0.0 [b]

* Fertilizing ability was determined by the number of fertile eggs / total no. of eggs laid during each week. Within column, values with different superscripts differ significantly ($P<0.01$) from each other.

Table 4. Duration of the maintenance of motility of bull, ram, boar and fowl spermatozoa incubated with cultured cells at 41°C in 5 % CO_2 + 95 % air

Cells in incubation medium	Duration of the maintenance of motility (hours)			
	Bull	Ram	Boar	Fowl
HeLa cells	15	15	15	120
BHK-21 cells	18	15	21	120
None (control)	6	6	27	24

Each figure represents an average of 5 trials.

Table 5. Duration of the maintenance of motility of bull, ram, boar and fowl spermatozoa incubated with cultured cells at 38°C in 5 % CO_2 + 95 % air

Cells in incubation medium	Duration of the maintenance of motility (hours)			
	Bull	Ram	Boar	Fowl
HeLa cells	18	27	24	120
BHK-21 cells	18	27	27	120
None (control)	9	9	27	24

Each figure represents an average of 5 trials.