The Importance of Survival of Oviducal Cells for Prolonged Viability of Fowl Spermatozoa

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(Received February 8, 1983)

Abstract Oviducal epithelial cells were cultured and the effects of living or dead cells on the survival of fowl spermatozoa were investigated. When spermatozoa were incubated at 41°C with living cultured cells from the shell gland, their motility, assessed at room temperature (20~25°C), was maintained for 6 days. Fertilizing ability was also retained for at least 2 days. In contrast, spermatozoa incubated with cultured cells killed by ultra-violet irradiation lost their motility and fertilizing ability rapidly. During the incubation at this temperature, the sperm motility declined along with the degeneration of living cultured cells, and a high correlation ($r=+0.85$) was observed between the number of living cells and sperm motility. These results suggest that the prolonged survival of fowl spermatozoa is dependent on living cultured cells.

Jpn. J. Zootech. Sci., 54 (10): 679-684, 1983

When fowl spermatozoa are stored in RINGER's solution at 41°C, normal body temperature, fertilizing ability is lost within a few hours¹⁾. However, when spermatozoa were incubated at the same temperature with fowl oviducal epithelial cells, they remained motile for 5 days and fertilizable for at least 4 days²⁾. Fowl spermatozoa incubated at 41°C with other sources of cultured cells were also found to be able to survive for long periods³⁻¹⁰⁾. Such cultured cells also contributed to the maintenance of motility of some mammalian spermatozoa⁷⁾.

It is reported that fowl spermatozoa incubated at 41°C with cultured oviducal cells are almost immotile whilst resting on the surface of the cells and their potential survival is maintained for long periods²⁾. It seems also possible that a substance(s) supplied by the cultured cells reaches the spermatozoa by diffusion and effectively prolongs their survival^{5,6)}. The present study reports that only living oviducal cells, but not dead cells, can maintain survival of fowl spermatozoa in culture.

Materials and Methods

The birds used were White Leghorns (Shaver strain) obtained from Koyu Poultry Farm, Miyazaki, Japan, and housed in cages under artificial light (14 hr) and fed *ad libitum* a proprietary breeder diet and water.

The cells obtained from the epithelia of the shell gland of the hen's oviduct were prepared for culture according to the methods described previously²⁾. The culture medium was made up by mixing nine parts of EAGLE's Minimum Essential Medium containing 6 mg kanamycin sulphate and 0.6 mg phenol red per 100 ml (Nissui No. 1, Nissui Pharmaceutical Co., Japan) with one part of heat-inactivated newborn calf serum (Commonwealth Serum Laboratories, Victoria, Australia). The pH of the mixture was adjusted to 7.2 with a 7.0% sodium bicarbonate solution. One ml of the cell suspension including 2×10^6 cells were seeded into small Petri dishes and cultured at 41°C in a CO₂ incubator (5% CO₂+95% air) by the stationary monolayer method.

In Exp. 1, the cells were cultured for 3 days. The cells proliferated and covered the bottom of one group of the dishes were killed by ultra-violet irradiation at intervals of 15 cm with 15 W germicidal lamp for 30 min, while the other group of the dishes was kept without irradiation. After discarding the medium and washing the surface of the cell mass with HANKS' solution¹¹⁾, newly prepared culture medium (1 ml) was added. The spermatozoa collected from several roosters by the method of FUJIHARA *et al.*¹²⁾, washed once with the medium by centrifuging ($500 \times g$, 10 min.) and resuspended in the medium were added to reach a sperm concentration of 2×10^8 /ml. The spermatozoa and cells were incubated as described above for the culture of cells alone. Spermatozoa incubated in culture medium without cultured cells were used as controls. Sperm motility was assessed at room temperature (20~25°C) at intervals during the incubation period at 41°C and scored on the 0 to 5 scale¹³⁾. The number of abnormal spermatozoa was determined on the 2nd and 4th day of incubation according to the procedures reported elsewhere²⁾. Fertilizing ability was examined using the spermatozoa incubated with cultured cells or in the cell-free medium for 2 days. Approximately 1.0×10^8 spermatozoa in about 0.5 ml culture medium were inseminated into the shell gland of pullet's oviduct by the method of BOBR *et al.*¹⁴⁾. From 2 days after the insemination, any eggs laid were collected, incubated and examined on the 4th day of incubation for fertility. The duration of fertility was also determined. The data obtained were analysed statistically by the method of DUNCAN¹⁵⁾.

In Exp. 2, the number of living cultured cells per ml was counted directly with a Burkner-Turk hemocytometer using trypan blue from 1 day after culture. On the 3rd day of culture, the culture medium (1 ml) was exchanged and one group of dishes continued to incubate without adding fowl spermatozoa. Spermatozoa were added to the other group of dishes to reach a sperm concentration of 2.0×10^8 /ml and further incubated with cultured cells. Sperm motility and percentage of abnormal spermatozoa were determined every day during the 7-day incubation period at 41°C as described above. Correlation coefficients between the sperm motility score and the number of living cultured cells and between the numbers of abnormal spermatozoa and of living cultured cells were measured according to the method of SNEDECOR and COCHRAN¹⁶⁾.

Results

Experiment 1. Effects of incubation with living or dead cultured cells on the motility and fertilizing ability of fowl spermatozoa

The changes of motility evaluated at 20~25°C during incubation at 41°C are shown in Table 1. Spermatozoa incubated with living cultured cells maintained their motility for 6 days. However, those incubated with dead cells and in cell-free medium (control) lost their motility rapidly. As shown in Table 2, the numbers of abnormal spermatozoa were also lower in the group cultured with living cells than with dead cells or without cells ($P<0.01$).

As shown in Table 3, the proportions of fertilized eggs by spermatozoa incubated with living cells during the first and second week were 56.3% and 32.1%, respectively. In contrast, spermatozoa incubated with dead cells or without cells (control)

Table 1. Motility score (at 20-25°C) of fowl spermatozoa incubated with living or dead cultured cells at 41°C in 5% CO₂+95% air

Cells	Days after addition of spermatozoa							
	0	1	2	3	4	5	6	7
Living	5.0	4.2	3.6	3.2	2.2	1.8	0.4	0.0
Dead	5.0	2.4	0.0	0.0	0.0	0.0	0.0	0.0
Cell-free (control)	5.0	1.8	0.0	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 abnormal fowl spermatozoa after incubation with living or dead oviducal cells

Cells	Incubation period	
	2 days	4 days
Living	19.2±1.4 ^a	33.2±5.9 ^d
Dead	85.3±1.6 ^b	95.5±0.7 ^c
Cell-free (control)	93.7±1.4 ^c	95.6±0.7 ^e

Within columns, values without a common superscript differ significantly ($P<0.01$) from each other.

Table 3. Fertilizing ability of fowl spermatozoa incubated with living or dead cultured cells for 2 days at 41°C in 5% CO₂+95% air

Cells	No. of hens inseminated	Fertilized eggs (%)		Mean duration of fertility (days)
		1st week	2nd week	
Living	5	56.3(18/32)*	32.1(9/28)*	7.2
Dead	5	0.0(0/34)	0.0(0/29)	0.0
Cell-free (control)	5	0.0(0/31)	0.0(0/29)	0.0

*Number of fertilized eggs/no. of eggs laid.

Fig. 1. Changes of the number of living cultured cells (mean $\pm$ S. E. M. of 5 trials) incubated with (O) or without (●) fowl spermatozoa at 41°C in 5% CO₂+95% air.

Fig. 2. Changes of sperm motility (●) and number of abnormal spermatozoa (O) incubated with cultured cells at 41°C in 5% CO₂+95% air. Values are mean $\pm$ S. E. M. of 5 trials.

had no fertilizing ability.

Experiment 2. The relationship between the number of living cultured cells and the sperm motility or the number of abnormal spermatozoa during incubation

The changes of the number of living cultured cells during incubation at 41°C are shown in Fig. 1. The cells proliferated rapidly covering the bottom of the Petri dish and their maximal number reached on the 2nd day of culture. Thereafter, the number of living cells decreased gradually when no spermatozoa were added. In contrast, when spermatozoa were added, rapid degeneration of the cultured cells was observed, especially up to the 3rd day after addition of spermatozoa.

The changes of sperm motility and the number of abnormal spermatozoa during incubation at 41°C are shown in Fig. 2. Spermatozoa incubated with cultured cells exhibited vigorous movement up to the 4th day. Then it decreased rapidly with lowered number of the living cells. There was a high correlation between the sperm motility score and the number of living cultured cells ($r=+0.85$). The number of abnormal spermatozoa increased proportionally to the decrease of the number of living cultured cells, and a high negative correlation between both phenomena was also observed ($r=-0.79$).

Discussion

The nutrients necessary for maintenance of survival of cultured cells or spermatozoa during incubations are quite limited in the present experimental condition. It is therefore considered that the incubation of spermatozoa alone is more favourable for the prolongation of their survival than in spermatozoa cultured with living cells. In the present study, however, the spermatozoa incubated with living cultured cells maintained their motility and fertilizing ability for long periods compared with those incubated with dead cells or in cell-free medium. As already suggested by ASHIZAWA and NISHIYAMA⁵⁾ and FUJIHARA and HOWARTH⁶⁾, the present results indicate that

substance(s) supplied by living oviducal cells may have a role(s) in prolongation of sperm survival. The maintenance of motility of spermatozoa incubated with dead cultured cells was similar to those of spermatozoa incubated in cell-free medium (control). Therefore, it is considered that dead cells will not release toxic products into the culture medium, but merely stop providing nutrients or other factors necessary for the survival of spermatozoa.

Furthermore, the present investigation showed that the cultured cells incubated in sperm-free medium were able to survive for long periods compared with those incubated with spermatozoa together (Fig. 1), while spermatozoa incubated in cell-free medium were not able to survive for long periods compared with those incubated with cultured cells (Table 1). This suggests that the cultured cells and spermatozoa are not in the interdependence for the maintenance of their survival, but that only the prolonged survival of spermatozoa depends on the presence of living cultured cells.

Acknowledgements

This study was supported in part by a grant from the Ministry of Education, Science and Culture, Japan (No. 56760175).

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培養卵管上皮細胞の生死が鶏精子の生存性 延長に及ぼす影響

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鶏精子の生存性が培養細胞の生死によって影響を受けるか否かを検討した。白レグ産卵鶏の卵管上皮から得た細胞を 41°C，5% CO₂+95% 空気の条件下で培養した。培養開始 3 日後に紫外線を照射し細胞を死滅させた区（死滅区）と，照射せず生存状態に保った区（生存区）を設定し，両区にそれぞれ 2×10^8 /ml の濃度になるように精子を添加して，41°C の温度条件下で保存した。その結果，死滅区の精子は 2 日後に室温での運動を停止したのに対し，生存区の精子は 6 日間運動性を維持した。2 日間保存の精子を人工授精した結果，死滅区の精子では受精率は得られなかったが，生存区の精子では 56.3% の受精率が得られた。つぎに，培養 3 日後の細胞に精子を添加した区（添加区）と添加しなかった区（無添加区）を

設定し，両区の生存細胞数を毎日測定すると同時に添加区における精子の運動性及び奇形率の推移を測定した。その結果，生存細胞数は培養開始 2 日後に最高になり，その後は徐々に減少したが，3 日後に精子を添加することにより急速に減少した。これに伴い精子の運動性は速やかに低下し，生存細胞数と精子の運動性との間に高い正の相関 ($r=+0.85$) が認められた。以上の結果から，鶏精子の生存延長には生きている培養細胞の存在が有効であり，さらに両者の関係は互いの生存を助け合う相互協力的なものではなく，一方的に精子が細胞に依存していると考えられた。

日畜会報，54 (10): 679-684, 1983