The mechanisms of reversible immobilization of fowl spermatozoa at body temperature

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Summary. Intact fowl spermatozoa became almost immotile at 40°C, but motility increased significantly at 30°C. The oxygen consumption at both temperatures was 8–11 $\mu\text{l O}_2/10^{10}$ spermatozoa·min⁻¹. The ATP concentration at 40°C was higher than that at 30°C but ADP concentration at 30°C was higher than that at 40°C. Consequently, the ATP/ADP ratio at 30°C (1.9–2.2) increased to 3.5–3.7 at 40°C. The motility of intact spermatozoa at 40°C was effectively restored by 2 mM-Ca²⁺, 10% seminal plasma and 10% peritoneal fluid taken at the time of ovulation. In contrast, these effectors did not restore the motility of demembranated spermatozoa at 40°C. Motility of demembranated spermatozoa was restored at 30°C. These results suggest that the immobilization of fowl spermatozoa at 40°C occurs due to a decrease in flagellar dynein ATPase activity. Furthermore, the action of effectors for motility such as Ca²⁺ may not be directly on the axoneme, but mediated by solubilized substances which have been removed by demembration of the spermatozoa.

Keywords: spermatozoa; motility; immobilization; temperature; fowl

Introduction

Generally, both vigour of motility and metabolic activity of mammalian spermatozoa increase with rising temperature. Maximum metabolic activity of spermatozoa occurs between 40 and 47°C (Beck & Salisbury, 1943; Freund *et al.*, 1959; Salisbury & Lodge, 1962). With fowl spermatozoa, in contrast, it has been reported that most synthetic diluents preserve the vigour of motility at room temperature but inhibit it at 40–41°C, normal body temperature (Munro, 1938; Nevo & Schindler, 1968; Ashizawa & Nishiyama, 1978; Takeda, 1982a; Wishart & Ashizawa, 1987). The immobilization is not permanent and movement resumes when the temperature is lowered to room temperature or calcium, caffeine, seminal plasma and female peritoneal fluid taken at the time of ovulation are added (Munro, 1938; Nevo & Schindler, 1968; Ashizawa *et al.*, 1976; Ashizawa & Nishiyama, 1978; Takeda, 1982a; Ashizawa & Okauchi, 1984; Ashizawa & Wishart, 1986, 1987; Wishart & Ashizawa, 1987). The motility-stimulating factors in seminal plasma and peritoneal fluid are believed to be calcium and a low molecular weight (~200), dialysable substance (Ashizawa & Wishart, 1986, 1987). However, the mechanisms for the immobilization and restoration of fowl sperm motility remain unknown.

It has been suggested that demembranated fowl spermatozoa (Ashizawa *et al.*, 1989) may be useful to clarify the mechanisms of the reversible, temperature-dependent immobilization of fowl spermatozoa. This study was undertaken to investigate this phenomenon.

Materials and Methods

Animals and semen collection. Male fowl (White Leghorn, Babcock strain) were obtained from Akagi Poultry Breeding Farm, Miyazaki, Japan. They were caged individually, given 14 h light/24 h and fed a commercial breeders ration *ad libitum*. Semen was collected by the method of Bogdonoff & Shaffner (1954).

Preparation of spermatozoa. Samples of semen pooled from 4–6 different males were diluted approximately 10-fold in 150 mM-NaCl with 20 mM-TES (*N*-Tris-[hydroxymethyl]-methyl-2-aminoethanesulphonic acid) at pH 7.4 and centrifuged at 700 *g* for 13 min at room temperature (20–25°C). The washed spermatozoa were reconstituted in the same buffer to give a final concentration of $\sim 1 \times 10^9$ cells/ml. Samples of 3–4 ml were poured into 30-ml Erlenmeyer flasks with a screw cap.

Measurement of motility, oxygen consumption and ATP and ADP concentrations of intact spermatozoa at 30°C and 40°C. The preparations were incubated aerobically in a shaking water bath at 30°C for 15 min, and then transferred to another shaking water bath and incubated at 40°C for 15 min. After that, they were re-incubated at 30°C for 15 min. Finally, they were moved to 40°C again and incubated for 15 min. Overall, the samples were incubated for 60 min at 30°C and 40°C, changing every 15 min. During incubation, sperm motility, oxygen consumption and ATP and ADP concentration were measured every 5 min at each temperature.

Sperm motility was assayed as described by Wishart & Ross (1985), using a double-beam spectrophotometer (Shimadzu, Model UV-150-02, Kyoto, Japan), but substituting the NaCl/TES buffer described above. The constant, $\%(\Delta OD)_m$, which correlates with the percentage of motile spermatozoa (Wishart & Ross, 1985), was used to describe 'motility'. Numbers of spermatozoa were established from the constant OD_m .

Oxygen consumption of spermatozoa was determined polarographically with a Clark electrode by the method of Kielley (1963), using a YSI model 53 biological oxygen monitor (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio, USA). The rate of oxygen consumption was expressed in terms of $\mu\text{l O}_2$ consumption/ 10^{10} spermatozoa·min⁻¹.

ATP concentration in spermatozoa was assayed in a boiled extract (Wishart, 1982). ADP concentrations were assayed by the method of Kimmich *et al.* (1975) and ATP/ADP ratios were calculated.

Motility of intact and demembrated spermatozoa incubated with various effectors at 30°C and 40°C. Intact spermatozoa incubated aerobically for 15 min at 40°C were demembrated at 40°C according to the method described previously (Ashizawa *et al.*, 1989). The extraction medium used consisted of 0.1% Triton X-100, 0.2 M-sucrose, 25 mM-potassium glutamate, 1 mM-MgSO₄, 1 mM-dithiothreitol and 20 mM-Tris-HCl buffer (pH 7.9). The reactivation medium consisted of 0.5 mM-ATP, 0.2 M-sucrose, 25 mM-potassium glutamate, 1.5 mM-MgSO₄, 1 mM-dithiothreitol and 20 mM-Tris-HCl buffer (pH 7.9). To examine the effectors for motility, various concentrations of CaCl₂, MgSO₄, seminal plasma and peritoneal fluid of hens taken at the time of ovulation were added to the reactivation medium. Seminal plasma and peritoneal fluid were prepared by the methods of Ashizawa & Wishart (1986, 1987). The suspension of demembrated spermatozoa was placed on a microscope slide chamber, and the motility of spermatozoa was recorded by videomicroscopy at 40°C on the thermostatically-controlled warm plate. The percentages of motile spermatozoa were measured by the method of Katz & Overstreet (1981). After that, the same samples were cooled to 30°C and the motility was observed again. The motility of intact spermatozoa which were suspended in the extraction medium without Triton X-100 and transferred to the reactivation medium was also measured by the same method. All data were subjected to statistical analysis by the method of Duncan (1955).

Results

Effects of temperatures on the motility, oxygen consumption, ATP and ADP concentrations of intact fowl spermatozoa

Sperm motility was markedly affected by the temperature (Fig. 1a). During the first incubation at 30°C, spermatozoa showed $\sim 50\%$ motility, and even after a second incubation at 30°C, $\sim 40\%$ motility was obtained. In contrast, spermatozoa lost motility quickly at 40°C.

The oxygen consumptions at both temperatures were 8.2–11.0 $\mu\text{l O}_2/10^{10}$ spermatozoa·min⁻¹ (Fig. 1b). There was no significant difference between the two temperatures ($P > 0.01$).

The ATP content (nmol/ 10^9 spermatozoa) was higher at 40°C (47.8–50.1) than at 30°C (38.3–43.3) and the ADP content (nmol/ 10^9 spermatozoa) was lower at 40°C (13.7–14.8) than at 30°C (19.1–21.9) (Fig. 2). Consequently, the ATP/ADP ratio at 30°C (1.9–2.2) increased significantly to 3.5–3.7 at 40°C ($P < 0.01$) (Fig. 2).

Fig. 1. The motility (a) and oxygen consumption (b) of intact fowl spermatozoa during incubation at 30°C (○) and 40°C (●). Each point represents the mean ($\pm$ s.e.m.) of 5 samples of spermatozoa. * $P < 0.01$ compared with 0 min value.

Fig. 2. ATP (○—●) and ADP (△—▲) concentrations and ATP/ADP ratio (□—■) of intact fowl spermatozoa during incubation at 30°C (○, △, □) and 40°C (●, ▲, ■). Each point represents the mean ($\pm$ s.e.m.) of 5 samples of spermatozoa. * $P < 0.01$ compared with 0 min value.

Effects of various effectors on the motility of intact and demembranated fowl spermatozoa at 40°C and 30°C

Motility became vigorous even at 40°C when intact spermatozoa were suspended in fluids to which had been added 2 mM- Ca^{2+} , 10% seminal plasma or 10% peritoneal fluid (Table 1). In contrast, the addition of 2 mM- Mg^{2+} was not effective. Motility of these samples of spermatozoa was restored at 30°C. However, at 30°C, no significant stimulation of motility by calcium, seminal plasma and peritoneal fluid was observed. These results were similar to those reported by Wishart & Ashizawa (1987). In contrast, these substances had no effect with the demembranated spermatozoa at 40°C (Table 1). In addition, cAMP was not effective. Motility of all samples of spermatozoa was restored at 30°C, although to different levels, depending on the concentrations of effectors, e.g. high concentrations of calcium (10 mM) inhibited motility.

Table 1. Effects of various substances on the motility of intact or demembranated fowl spermatozoa at 40°C and 30°C

Spermatozoa	Substances (conc.)	Motility (%)	
		40°C	30°C
Intact	None (control)	8.7 ± 0.6 ^a	62.5 ± 3.2 ^a
	Ca^{2+} (2 mM)	68.2 ± 2.3 ^b	70.3 ± 2.4 ^a
	Mg^{2+} (2 mM)	9.0 ± 1.2 ^a	63.4 ± 4.1 ^a
	Seminal plasma (10%)	71.8 ± 3.2 ^b	70.6 ± 4.8 ^a
	Peritoneal fluid (10%)	72.4 ± 4.1 ^b	71.7 ± 3.2 ^a
Demembranated	None (control)	0	49.4 ± 3.0 ^a
	Ca^{2+} (0.1 mM)	0	42.6 ± 5.0 ^{ab}
	(1 mM)	0	32.3 ± 4.6 ^{bc}
	(10 mM)	0	19.0 ± 4.0 ^d
	Ca^{2+} (1 mM) + cAMP (10 µM)	0	35.4 ± 4.0 ^{bc}
	Mg^{2+} (0.1 mM)	0	46.9 ± 3.5 ^a
	(1 mM)	0	39.1 ± 4.3 ^{ab}
	(10 mM)	0	24.1 ± 3.6 ^{cd}
	Mg^{2+} (1 mM) + cAMP (10 µM)	0	58.3 ± 2.3 ^a
	Seminal plasma (0.1%)	0	43.9 ± 3.4 ^{ab}
	(1%)	0	56.6 ± 3.6 ^a
	(10%)	0	62.7 ± 6.6 ^a
	Peritoneal fluid (0.1%)	0	39.1 ± 3.5 ^{ab}
	(1%)	0	44.8 ± 7.0 ^{ab}
	(10%)	0	58.2 ± 5.7 ^a

Each figure represents the mean (± s.e.m.) motility of 5 samples of spermatozoa.

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

Discussion

The present results showed that, as in previous studies (Ashizawa & Nishiyama, 1978; Takeda, 1982b; Wishart, 1984), the oxygen consumption of intact spermatozoa did not increase at 40°C compared with that 30°C. Therefore, it is assumed that the amounts of ATP production by respiration were similar at both temperatures. Nevertheless, the ATP/ADP ratio at 40°C was about 1.8-fold higher than that at 30°C (Fig. 2). In addition, the demembranated spermatozoa were completely quiescent at 40°C in spite of containing a high concentration of ATP in the reactivation medium, and their motility was restored at 30°C. It is well known that flagellar movement of

spermatozoa is based on the active sliding of microtubules as a result of ATP hydrolysis of dynein ATPase (for reviews, see Satir, 1979; Warner & Mitchell, 1980). Therefore, it is possible that for fowl spermatozoa activity for ATP hydrolysis to ADP is lower at 40°C than at 30°C. That is, fowl spermatozoa might be quiescent due to a decrease in flagellar dynein ATPase activity, although the effects of temperature on the activity of dynein ATPase of fowl spermatozoa were not measured in this experiment. Further study is therefore needed to examine the difference of dynein ATPase activity of spermatozoa from fowl and other species.

Motility became vigorous even at 40°C when intact fowl spermatozoa were suspended in fluid to which calcium, seminal plasma or peritoneal fluid had been added (Table 1). These results were similar to those reported previously (Ashizawa & Wishart, 1986, 1987; Wishart & Ashizawa, 1987). In contrast, these were not effective with the demembranated spermatozoa at 40°C. Cyclic AMP also did not affect motility (Table 1). The spermatozoa did not degenerate due to the high temperature treatment (40°C) during demembranation, because motility of the same samples of spermatozoa was restored at 30°C. From these results, we suggest that the action of substances such as calcium that affect motility is not directly on the axoneme, but is mediated by the solubilized substances such as membrane-associated enzymes which have been removed by the demembranation of the spermatozoa.

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