Stability of the sperm plasma membrane of hibernating bats (*Myotis velifer*) compared with other mammals

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Previous experiments have established that the long-lived spermatozoa of hibernating bats are resistant to the acrosome reaction and fertilization *in vitro* using conventional techniques. We tested the hypothesis that the membranes of these spermatozoa are more resistant to perturbation than those of other mammals. We exposed them to non-specific bilayer destabilizing agents and abrupt changes in incubation temperature and tested their response by observing their status (motility and viability) after a time interval compared with other mammals (golden hamster, rabbit, human). The results did not support the hypothesis. The inherent longevity of bat spermatozoa may thus be a function of some component other than unique resilience of their plasma membrane.

Introduction

It has long been known that the spermatozoa of many species of bats are stored for many months, their prolonged viability far exceeding that of any other mammals (Wimsatt, 1942, 1944; Racey, 1973, 1975, 1979). They are stored in the caudae epididymides of males as well as in the uterus and uterotubal junction of females (Hiraiwa and Uchida, 1956a, b; Krutzsch *et al.*, 1982; Uchida and Mori, 1987). For most of the bat species displaying this phenomenon, storage largely coincides with hibernation, but limited storage of spermatozoa has also been described in some tropical heterothermic species that do not hibernate (Gopalakrishna and Madhavan, 1971; Medway, 1972). Thus, low temperature cannot be solely responsible for the unusual longevity of bat spermatozoa. Either the spermatozoa themselves or their storage organs must be modified in some way to provide for this unique situation.

Research has sought to understand the biology of sperm longevity in bats by morphological (Fawcett and Ito, 1965; Uchida and Mori, 1972; Racey, 1975; Krutzsch *et al.*, 1982; Mori *et al.*, 1982; Uchida *et al.*, 1984), biochemical (Crichton *et al.*, 1981, 1982; Krutzsch *et al.*, 1984) and experimental (Lambert, 1981; Uchida and Mori, 1987) approaches. However, although several theories have been offered to explain sperm storage, none has been unequivocally substantiated.

We have established that bat spermatozoa resist capacitation and the acrosome reaction when cultured under conditions (Yanagimachi, 1988) that are known to induce these events in other mammals; similar observations were made after experimental variations upon established procedures (Krutzsch and Crichton, 1991). Furthermore, contrary to the observations of Lambert (1981), *in vitro* fertilization tests using the hamster zona-pellucida-free oocyte test failed to reveal decondensing

bat sperm heads within hamster ooplasm, even after exposure to the calcium ionophore A23187 and various proteases. That a normal mammalian acrosome reaction does occur in bat spermatozoa, however, is shown by the occurrence of vesicular membranes, on occasion, after the exposure of spermatozoa to the calcium ionophore A23187 or to bat oocyte–cumulus complexes. Together these observations suggested that further attempts to produce *in vitro* fertilization with bat spermatozoa should be suspended until more knowledge accumulates on the underlying mechanisms for their unique longevity.

The unusual longevity, 'stability' and resistance of bat spermatozoa to *in vitro* fertilization (IVF) could be due to (i) unique resilience of the plasma membrane of bat spermatozoa, thereby eliminating preparation of the sperm surface before effective interaction with the oocyte or (ii) failure to provide a critical factor(s) in the incubation medium. A unique species specificity of the hamster oocyte may lead to exclusion of bat spermatozoa. To test the first possibility, a series of experiments was performed to test the tolerance of bat spermatozoa to environmental conditions that would perturb their plasma membrane. We compared the viability of bat spermatozoa with that of the hamster, human and rabbit in various *in vitro* conditions, to assess whether bat spermatozoa are unique in their response.

Materials and Methods

Culture media

BWW medium (Biggers *et al.*, 1971) containing 0.3% bovine serum albumin (BSA: Fraction V, Sigma, St Louis, MO) was used for all experiments. Unless otherwise stated, all chemicals were purchased from Mallinckrodt Chemical Company (St Louis, MO) or Sigma Chemical Company (St Louis, MO).

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Table 1. Minimum concentration of various membrane-destabilizing reagents at which 100% of spermatozoa of four mammals were immobilized in 5 min

Reagent	Concentration range tested	Spermatozoa of:			
		Hamster	Bat	Rabbit	Human
Triton X-100	0.001–0.1%	0.02	0.02	0.03	0.01
Hyamine	0.001–0.1%	0.06	0.06	0.04	0.01
Lauryl sulfate	0.1–2.0 mmol l ⁻¹	1.0	1–2	1.0	1.0
Polymyxine	0.1–3.0%	1.0	1.0	3.0	2.0
Tetracaine	1.0–3.0 mmol l ⁻¹	2.0	3.0	> 3.0	2.0
Digitonin	0.01–2.0%	0.1	0.1	0.1	0.1
Saponin	0.0001–1.0%	0.001	0.001	0.1	0.1
Nystatin	0.0001–1.0%	0.01	0.01	0.01	0.01
Acetone/methanol	10–50%	35	40	35	50

Collection of spermatozoa

Adult male bats (*Myotis velifer*) were collected from the wild in Arizona in the autumn (September–October 1989). The testes were atrophying at this time and all the spermatozoa had been transported to the caudae epididymides where they are stored until the following spring. For several weeks after capture, animals were kept at room temperature and food (larval *Tenebrio molitor*) and water were provided *ad libitum* until they had accumulated sufficient body fat reserves to hibernate. Bats were then placed in an artificial hibernaculum (15°C), where they were held until required for experiments.

Caudae epididymides were removed from etherized bats, wrapped in saline-moistened gauze within a tightly capped disposable microcentrifuge tube and placed in a styrofoam box with ice for overnight shipment to Hawaii. The inside temperature of the box was 6–10°C upon arrival. The tubes containing the epididymides were then placed in a refrigerator (4°C) until the start of an experiment that day (i.e. about 24 h after collection of epididymides).

Spermatozoa were liberated from the caudae epididymides of bats, Syrian (golden) hamsters and rabbits by puncturing the epididymal tubules with a fine needle. Human ejaculated spermatozoa, which were obtained through a human IVF clinic, were washed and centrifuged (500 g, 5 min) to remove seminal fluids and debris. The 'swim-up' method (Overstreet *et al.*, 1979; Cummins and Yanagimachi, 1986) was used for the collection of highly motile populations of hamster and human spermatozoa. Populations of spermatozoa (about $1-5 \times 10^7$) from all species were estimated to be at least 80% motile at the start of all experiments.

Effect of temperature shock

To examine the effect of temperature shock on sperm viability, sperm suspensions were placed in narrow glass microcentrifuge tubes (approximately 2 mm internal diameter, 200 µl volume), and transferred rapidly between warm (37°C) and cold (0°C) water baths. The duration of each exposure to warm and cold temperature was 5–10 min; 1–5 successive exposures (2–10 transfers) were made before spermatozoa were examined.

Effect of plasma-membrane-destabilizing agents on the survival of spermatozoa

The following membrane-perturbing reagents were prepared in BWW immediately before use:

(1) Triton X-100 (0.001–0.1%), hyamine 2389 (Rhom and Haas Co.) (0.001–0.1%) and lauryl sulfate (0.1–2 mmol l⁻¹). These detergents are known to disorganize membrane phospholipids.

(2) Polymyxine (0.1–3.0%), a chemical that enters lipid bilayers and increases membrane permeability (Pache *et al.*, 1972).

(3) Tetracaine (1.0–3.0 mmol l⁻¹), a chemical that enters lipid bilayers to stabilize membrane lipids at low concentrations (< 1 mmol l⁻¹) and induce membrane lysis at high concentrations (> 1 mmol l⁻¹) (Butler *et al.*, 1973).

(4) Digitonin (0.01–2.0%), saponin (0.0001–1.0%) and nystatin (0.001–1.0% – dissolved initially in dimethyl sulfoxide), reagents that bind to sterols and disrupt membranes (Sutton *et al.*, 1961; Kinsky *et al.*, 1966; Addinck *et al.*, 1972; Elliot and Haas, 1976).

(5) A 1:1 mixture of acetone and methanol (10–50%), a lipid solvent.

Equal volumes (15 µl) of a sperm suspension and a test solution were mixed and incubated together for 5 min at room temperature (25°C). At the end of this period, sperm motility and viability were assessed.

Survival (motility) of spermatozoa

The sensitivity of sperm plasma membranes to the chemicals tested was evaluated by determining the minimum concentration at which 100% of spermatozoa were immobilized in 5 min. The survival of spermatozoa was assessed subjectively by viewing the drops of sperm suspension using phase-contrast or dark-field microscopy. Viability of immobilized spermatozoa was tested by mixing equal volumes of a 1% solution of Trypan Blue in Dulbecco's phosphate-buffered saline and the sperm suspension on a glass slide and immediately counting the proportion of live (unstained) to dead (stained) cells. At least

three replicates of each experiment were run using different males.

Results

Resistance of spermatozoa to temperature shifts

Rapid transfer of sperm samples between cold (0°C) and warm (37°C) temperatures did not greatly affect the viability of bat, rabbit or human spermatozoa. At least 70% of these spermatozoa were motile and alive even after exposure for five times to each set of temperatures (10 transfers). Under the same conditions, no more than 25% of hamster spermatozoa were alive.

Resistance of spermatozoa to membrane-destabilizing agents

Bat spermatozoa showed no greater tolerance to membrane-active reagents than the spermatozoa of the other mammals tested (Table 1). They were immobilized at similar or even lower concentrations.

Discussion

In the present study, we looked to the plasma membrane as a possible source of difference between spermatozoa of bats and those of other mammals. It is known that the lipid composition of the sperm plasma membrane differs considerably among species (Darin-Bennett and White, 1977; Peterson and Russell, 1985; Hinkovska *et al.*, 1986; Nikolopoulou *et al.*, 1986; Parks *et al.*, 1987; Agrawal *et al.*, 1988). It appears that the cholesterol:phospholipid ratio of the sperm plasma membrane may underlie species-specific differences in resistance to environmental stresses such as cold shock (Wales and White, 1959; Darin-Bennett and White, 1965; Darin-Bennett *et al.*, 1974). The time needed for capacitation of bat spermatozoa may be influenced by the lipid composition of the sperm plasma membrane, since the removal of cholesterol from the sperm plasma membrane has been implicated as an important step in this process in other species (Davis, 1978, 1981; Davis *et al.*, 1979; Go and Wolf, 1983; Langlais and Roberts, 1985; Suzuki and Yanagimachi, 1989; Parks and Ehrenwald, 1990).

The results of this study did not support our hypothesis that bat spermatozoa, by virtue of their inherent longevity, their ability to resist extreme temperature conditions *in vivo* and their resistance to our attempts to induce the acrosome reaction *in vitro*, are more tolerant to environmental stresses than are the spermatozoa of other mammals. Although bat spermatozoa displayed a much greater resilience than hamster spermatozoa to the imposition of all (except saponin) membrane-destabilizing agents and abrupt temperature shifts, they performed no better than rabbit or human spermatozoa under all experimental conditions tested. No explanation can be offered for the greater susceptibility of hamster and bat spermatozoa to saponin; there is no information for the membrane-destructive effects of this detergent that might distinguish between plasma membrane components. The observation that both human and rabbit spermatozoa showed a high degree of tolerance to temperature

shock is consistent with the data of Wales and White (1959). Human and rabbit spermatozoa have a higher cholesterol content (Darin-Bennett and White, 1977) than do spermatozoa of mammals that are more susceptible to temperature shock (e.g. bull, ram).

Despite our failure to demonstrate a difference between the spermatozoa of bats and those of other mammals under the conditions tested, it remains apparent, from our IVF trials, that bat spermatozoa are unique. Although it is possible that zona-free hamster oocytes are not penetrated by bat spermatozoa, it is apparent from electron microscopy that the acrosome reaction of bat spermatozoa is difficult to induce *in vitro*, at least under conditions that are sufficient for most other species. Capacitation, the acrosome reaction and egg penetration may require the provision of some critical component in the medium that has yet to be identified for this system. Mate and Rodger (1991) have noted a similar stability of the sperm acrosome of some marsupial species which also resist efforts to induce the acrosome reaction.

Although the present study failed to demonstrate a uniqueness of bat spermatozoa, the conditions and agents used may have failed to detect delicate, yet important, differences in membrane characteristics between bat spermatozoa and those of other mammals. Accordingly, studies to measure fine changes in sperm biology before immobilization are in progress, using rheological and flow cytometry technology.

Within the limitations of the data collected, the results of these experiments suggest that the spermatozoa of hibernating, sperm-storing bat species may not be modified in terms of the lipid composition of their plasma membrane. Rather, their prolonged viability may reflect the presence of some other membrane specialization (e.g. a glycoprotein) that must be removed or altered during modification of the plasma membrane for fertilization (Yanagimachi, 1988). Alternatively, specialized conditions in the storage sites in the male and female reproductive tracts may act in some way to maintain the life of the spermatozoa of sperm-storing species of bats.

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