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Comparative Study on Components and Activities of Sperm Head Plasma Membrane in Active and Hibernating Animals

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활동 및 동면동물의 정자 두부 Plasma Membrane의 성분 및 활성에 관한 비교 연구

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ABSTRACT

Fertilization pattern of north temperate bats is known to be unique for their sperm storage in the female reproductive tract during hibernation (e.g. Korean greater horseshoe bats). They copulate in fall but their ejaculated spermatozoa survive until the next spring. In another words they can persist to survive during long hibernation under the cold condition (8~13°C) and are to be fertilized with the ovum ovulated in the next spring, so called delayed fertilization. The present study was designed to observe morphological and functional changes of spermatozoa plasma membrane of the bats, hamsters which are hibernators, and mice which are non-hibernators in the room and the cold (bat-hibernation) temperatures and to confirm influence of the temperature on spermatozoa; survival rate, acrosome reaction rate, protein distribution, Na^+-K^+-ATPase activities and scanning electron microscopic histochemistry.

Based on the experimental results obtained in the present study, there were no significant morphological and functional differences in the spermatozoa plasma membrane in both the room and cold (bat-hibernation) temperatures and such an absence of difference suggests that the spermatozoa plasma membrane might play a pertinent role as a protector for consistent fertilization during and after hibernation.

Key words : Bat, Spermatozoa, Plasma membrane, Fertilization, Hibernation, Acrosome

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INTRODUCTION

It has been known that prior to fertilization of the spermatozoa and the ovum, two physiological steps (capacitation and acrosome reaction) take place always in the most species of mammals (Takeshi et al., 1986; Ali et al., 1994). These two physiological procedures are essential for assuring plasma membrane fusion, and the spermatozoa in the semen ejaculated should also keep their vigorous motility for success of fertilization. The acrosome reaction is characterized by breakdown of the outer membrane of the acrosome to form a number of pores through which the hyaluronidase, zonalysin and trypsin-like substance are to be liberated to the zona pellucida and the corona radiata of the ovulated ovum (Kohn et al., 1997).

Generally spermatogenesis of sperm storage typed bat such as hibernating Korean greater horseshoe bats is active in late autumn and their ejaculated spermatozoa have destined to be storaged in the female reproductive tract for 6 months or more, and maintained the capacitation until the next spring when ovulation and fertilization begin and carry through to completion (Mori et al., 1982; Uchida et al., 1984; Oh et al., 1985; Krutzsch et al., 1986). On the other hand, it has been reported that the spermatozoa remained in the ductus epididymidis have persisted to be alive during hibernation (Bernard, 1984).

During hibernation the storaged spermatozoa in the female reproductive tract are found embedded within the oviductal epithelium and the utero-tubal junction, which are morphologically characterized by tight adhesion of both the epithelial cell plasma membrane and the sperm head plasma membrane (Krutzsch et al., 1982). Such an intimate relationship of two plasma membranes strongly suggest that there might be apossibility of close relation between the plasma membranes and the nutrients for their survival during hibernation. Actually there have been reported some experimental evidences to support this possibility; existence of numerous glycogen granules in the uterine epithelial cells of the house bats (Pipistrellus), and inclusion of fructose in the uterine blood plasma of the little brown bats (*Myotis lucifugus*).

There have been reported some experimental results explaining this peculiar physiological penomenon of sperm storage, however the exact reason for this mechanism has not yet been clarified up to date as far as the hibernating bats are concerned, with special regard to their plasma membranes that migh be closely relatedto nutrient exchange. Crichton et al. (1993) has tried an experiment to examine plasma membrane resistance to temperature change between bats and other mammalian species, deducing that the bat plasma membrane might have a certain level of endurance to environmental unstable factors such as temperature fluctuation during hibernation. But he failed to find any specific differences from those of the other mammalian species.

The present study has been designed in order to examine the working hypothesis that persistence power of bat plasma membrane might be closely related toits components and activity during hibernation such as acrosome reaction, protein level and enzymatic activities, and thus performed to the spermatozoa kept in both conditions the room temperature and the hibernating temperature. On the other hand, those physiological parameters of a hibernator (hamster) and a non-hibernator (mouse) have also been examined for comparative analysis after the same experimental treatments in the present study.

MATERIALS AND METHODS

1. Experimental Animals

Bats (Korean greater horseshoe bat, *Rhinolophu ferrumequinu Korai*) used in the present study were captured in the Kumsong Gold Mine (abandoned) and lime caves; active bats captured in July and hibernation-preparing bats in October, hibernating bats in January. Two species of mammals, male mice (ICR

strain) and male hamster (Cyrian strain) were also used in the present study.

The active bats were caged in the iron-wired net box under the room temperature (20~25°C) and the bats captured before hibernation were kept in the cold room (8~13°C). Humidity of the atmosphere of the bat room and the cages were always kept in 80% for preventing their sensitive response to thirst.

The hamsters and the mice were also divided into two groups; room temperatureand cold (8~13°C) temperature and were kept at least for two weeks.

2. Preparations of Spermatozoa

The testes, the epididymis and the ductus deferens were respectively removed from the experimental animals and teased, and put into the incubation media (HAMs-F10), and incubated for 10 minutes under 37.5 °C in 5% CO_2 conditions (conical tube, Falcon, USA). After the incubation, the supernatants were washed by means of two-step Percoll gradient method using 80% and 40% solution, and then centrifuged at 300 g for 20 minutes (IEC, Centra-8R) and the specimens were storaged in 5% CO_2-incubator at 37.5°C.

3. Experimental Methods

1) Density Measurement of Spermatozoa

The density of the spermatozoa in the semen were calculated by means of haemocytometry (Mortimer et al., 1989), in a regular tetragon of which number of spermatozan heads were counted to take an average of 5 tetragons.

2) Survival Rate of Spermatozoa

For ascertaining spermatozoan survival, the Eosin-Nigrosin Test which is known as a routine method was applied in the present study. The Eosin-Nigrosin staining solution was made as follows; 0.67 g of Eosin Y was melted in 100 ml of distilled water, and then the solution was slowly heated and 10 g of Nigrosin was added before the solution boiled. The staining solution was filtered by filtering apparatus (Corning Co., New York, USA) and was kept in 4°C. Just prior to using, the staining solution was heated to room temperature for better staining.

When using, the same volume of the semen specimen and the Eosin-Nigrosin staining solution were mixed and one or two drops were threw down on the slide for staining. Negative (white coloured) staining of the spermatozoa heads was reckoned as alive state of a spermatozoon, and to the contrary positive (pink or red coloured) staining as dead state of it.

3) Analysis of Acrosome Reaction Rate

Acrosome reactin rate was examined by means of Triple Staining Method (Talbot et al., 1981); After staining the spermatozoa with 1.5% trypan blue (37°C for 15 minutes), 2% Bismark brown Y (40°C for 5 minutes) and 0.8% rose bengal (room temperature for 27 minutes) staining solution, the specimens were washed, then followed bt dehydration clearing and finally dried for one full day for microscopic observations. For discriminating live or dead spermatozoa, the classification method proposed by Talbot et al. (1981) was applied; the spermatozoa the acrosome of which were stained in pink colour were classified as live, and non-stained (white in colour) and degenerated as dead. Stainability and morphological characteristics of the postarosomal regions of the spermatozoa were also referred for the discrimination.

4) Protein Analysis

For protein analysis of the spermatozoa, the electrophoresis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis, SDS-PAGE) was applied and the fluid neccessary for the electrophoresis was prepared by the modified Laemmli's method Laemmli, 1970).

After run, the gel was stained by comassie brilliant blue for 30 minutes and decolourized by the mixture fluid (methyl alcohol 10, glacial acetic acid 10, and distilled water 80) for 3 hours. The specimens were dried by gel-dryer (Bio-rad 583 gel dryer, Bio-rad

Laboratories Co., USA) and finally photographed for analysis.

5) Activities of Na^+-K^+-ATPase

For measurement of Na^+-K^+-ATPase activities in the plasma membranes of the spermatozoa, electron microscopic histochemistry was applied in the present study, which has been demonstrated by McGrady (1979). The centrifuzed spermatozoa was put into the mixture fixative (2% paraformaldehyde and 0.5% glutaraldehyde) and coagulated by agarose gel for preventing disperse of the spermatozoa in the 0.1 M cacodylate buffer solution (pH 7.2) for one hour. After washing for 20 minutes respectively, the specimen was immersed in the substrate solution (Mayahara et al., 1980) in room temerature for 15 minutes at pH 8.8~9.2. After washing with the same buffered solution, the specimen was postfixed with 1% osmium tetroxide and washed again for 15 minutes respectively.

After routine treatments for dehydration, embedding and polymerization were performed, the specimens were sectioned, and stained with 1% toluidin blue solution, and finally thin sectioned for electron microscopic observations (JEM-1200EX II, JEOL Co., Japan) and photographed.

6) Ultrastructural Observations

For scanning electron microscopic observations, after finishing the same dehydration procedure with the former electron microscopic histochemistry, the specimens were dried by means of C.P.D. (critical point dryer, HCP-2, Hitachi, Japan) and put in the chamber fulled with liquid CO_2 in 20°C for 20 minutes and in 38 °C for 5 minutes, and then slowly cooled by 15°C. Finally the specimen was coated with gold (Ion sputter, E-1010, Hitachi, Japan) in 300 Å thickness for 6 minutes, and observed with a scanning electron microscope (Hitachi S-800, Japan) and photographed for analysis.

4. Statistic Treatments

Experimental data of the Eosin-Nigrosin test and the triple staining method for the survival rate and the acrosome reaction rate of spermatozoa were verified in 5% significant level using independent t-test.

RESULTS

1. Density of the Spermatozoa

Numeral density of spermatozoa exudated from the testes of each experimental animals was analyzed by means of haemocytometry; the density of spermatozoa in the Korean greater horseshoe bat was 2.0×10^6 sperm /ml under both cool and room temperature conditions, the hamsters 2.5×10^6 sperm/ml, the albino rats 2.1×10^6 sperm/ml.

2. Survival Rate of Spermatozoa

The survival rate of spermatozoa in the bat was 79.1% in the room temperature and 81.7% in the cold temperature (Table 1). This result showed that the spematozoa survival has not been influenced by the environmental changes. In the hamsters, the survival rate (82.0% in the room temperature and 81.5% in the cold temperature) and in the mice, the survival rate (81.3% in the room temperature and 78.8% in the cold temperature were not significantly changed respectively.

3. Analysis of Acrosome Reaction

The acrosome reaction rates in the experimental animals under the room temperature and the cold temperature were as follows (Table 2); the Korean greater

Table 1. Survival Rate (%) of Spermatozoa of the Bat, Hamster and Mouse at Room and Cold Temperatures

Animal Species	No. of Animals	Acrosome Reaction Rate (%) at Room Temp.	Acrosome Reaction Rate (%) at Cold Temp.
Bat	10	21.4+2.3*	21.9+3.6
Hamster	10	21.8+3.2	20.1+2.5
Mouse	10	20.2+2.2	20.1+2.4

* Mean+SD, $P>0.05$
No significant difference is found respectively between the survival rate at the room and cold temperature.

Table 2. Acrosome Reaction Rate (%) of Spermatozoa of the Bat, Hamster and Mouse at Room and Cold Temperatures

Animal Species	No. of Animals	Survival Rate (%) at Room Temp.	Survival Rate (%) at Cold Temp.
Bat	10	79.1+4.4*	81.7+4.1
Hamster	10	82.0+4.4	81.5+4.9
Mouse	10	81.3+2.5	78.8+3.2

* Mean+SD, $P>0.05$
No significant difference is found respectively between the acrosome reaction rate at the room and cold temperature.

horseshoe bats (21.4% and 21.9% in the room and the cold temperatures respectively, hamsters (21.8% and 20.1% in the room and the cold temperatures), and the mice (20.2% and 20.1% in the room and the cold temperatures respectively.

This analysis of acrosome reaction resulted in a no significant difference between the room temperature and the cold temperature as far as the triple staining method (Talbot et al., 1981) for spermatozoa was concerned.

4. Analysis of Protein Distribution

The protein distribution was examined by means of SDS-PAGE method and the following results have been obtained; the Korean greater horseshoe bats (a thick band of 65 kDa in both the room and the cold temperatures, and the others 54, 42, 33, and 29 kDa bands) (Fig. 1a), the hamsters (a thick band of 65 kDa and the others 39, 37, 36 kDa bands respectively) (Fig. 1b), and the mice (the same bands of 110, 65, and 39 kDa bands respectively) (Fig. 1c).

5. Na^+-K^+-ATPase Activities

The Na^+-K^+-ATPase activities was examined by means of electron microscopic histochemistry and the following observations were obtained; the intact spermatozoa of the Korean greater horseshoe bats had a

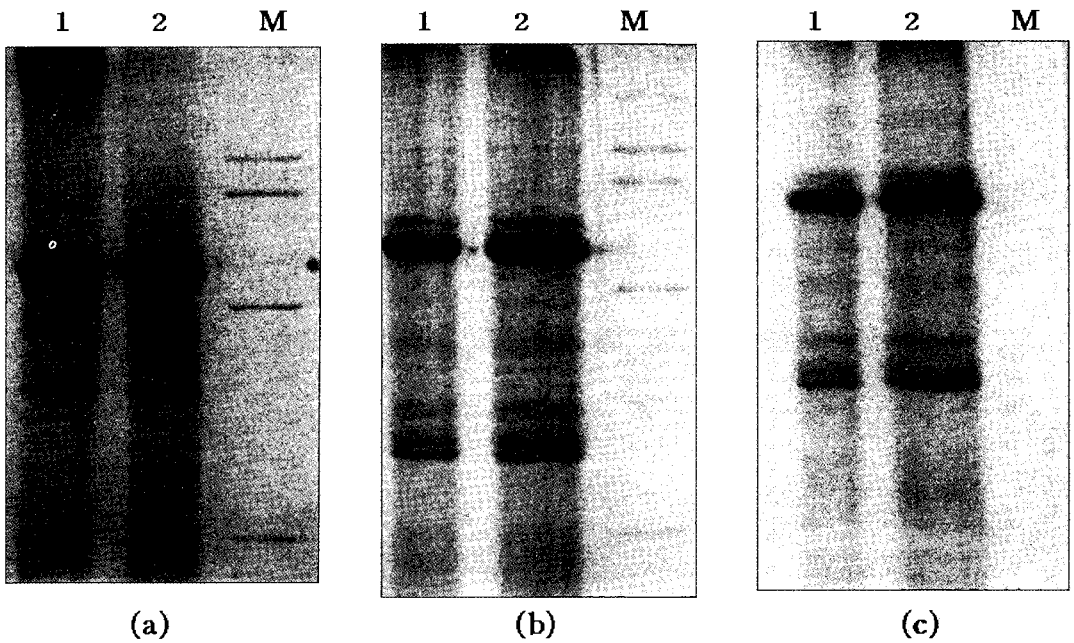

Fig. 1. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoretic Analysis of Proteins in Spermatozoa of the Bat (a), Hamster (b), Mouse (c).
Lane 1: Cold temperature, Lane 2: Room temperature, Lane M: marker protein (29 kDa: carbonic anhydrase, 39.8 kDa : alcohol dehydrogenase, 58.1 kDa: catalase, 97.4 kDa: phosphorylase B, 116 kDa: β-galactosidase, 205 kDa: α2-macroglobulin.

elongated sharp head (4.5 μm in length) with a tiny acrosome, smooth outer plasma membrane, and middle piece filled with well developed mitochondria (Fig. 2). However the substrate-treated spermatozoa had a tortuous outer plasma membrane and swollen acrosome which were thought to be positively reacted portions with substrates (Fig. 3a in the cold temperature and Fig. 3b in the room temperature). On the other hand, the substrate-treated spermatozoa of both the hamsdters and the mice also showed almost the same histochemical activities with those of the Korean greater horseshoe bats although some cytological differences were observed in the acrosome and the plasma membranes (Figs. 4a, b, 5a, b).

6. Ultrastructural Observations

The intact and substrate-treated spermatozoa of the Korean greater horseshoe bats were examined by the scanning electron microscopy; their heads were characterized by columnar in shape, smooth surface and blunt acrosomal edge in the intact spermatozoa (Fig. 6). The Na^+-K^+-ATPase-reacted sustrate (S) were visible on the head surface of the spermatozoa in both the room (a) and cold (b) temperatures as rough and white residuals. Their middle pieces were also peculiar in their outer bumpy surface bulged by mitochondria (Fig. 7a, b).

DISCUSSION

There have been reported several research papers on clarifying mechanism of fertilization, especialy its delaying of spermatozoa in the female reproductive tract; Beasley (1984) reported that the delaying might be due to length of photoperiod, and Pevet et al. (1981) demonstrated that pineal gland invoked hypothalamus to suppress reproductive function. Recently Crichton et al. (1993) reviewed widely the possible mechanisms of the delayed fertilization of spermatozoa.

There are several some research papers of spermatozoa survival rates; Crichton et al. (1994). demonstrated that although hibernating bat (Myotis velifer) spermatozoa are repeatedly invoked to be put into abrupt temperature change from 0°C to 37°C, the spermatozoa revealed much higher survival rate (70%) than those (25%) of hamster treated with the same ways. On the other hand, Krutzsch (1975) explained that in the canyon bat (Pipistrellus hesperus), early spring right after hibernation the Leydig cells became degenerated to be decreased in hormnonal secretions. This suggests that the blood plasma testosterone may be decreaed in amount to reveal a certain possibility of spermatozoa survival in the ductus epididymidis during hibernation.

Based on above-mentioned reports, the present study was designed in order to clarify influence of temperature changes (room and cold) on spermatozoa survival rate, acrosome reaction, protein distribution, Na^+-K^+-ATPase, and ultrastructure in hibernators, Korean greater horseshoe bats and hamsters, and non-hibernator mice.

In the survival rate analysis of the Korean greater horseshoe bats, no significant difference was found in the cold and room temperatures, and this result suggests that the spermatozoa are destined to be persistent without any environmental change as far as the temperature is concerned. Also in the hamsters treated with the cold and room temperatures, the spermatozoa survival rates were not influenced any more.

From experimental results of the survival rate analysis, it is deduced that the temperature changes (room and cold) could not influence the survival rats of the spermatozoa in the Korean greater horseshoe bats, hamsters, and the mice.

In the acrosome reaction analysis, no significant difference between experimental animals (Korean greater horseshoe bats, hamsters and mice) in the room and cold temperatures were found in the present study. This result suggests that the acrosome reaction of those animals is not susceptible to be degenerated as far as the environmental temperature changes are concerned.

As mentioned by Khalida et al. (1997), it was expect-

ed to find a certain level of difference in the protein distribution, but there were no differences between both the room and cold temperatures in the experimental animals. From the experimental results obtained in the protein distribution analysis, it is deduced that there were no specific difference of protein distribution in the room and cold temperatures.

Recently Khalida et al. (1997) reported that in many mammals including man, PH-20 protein in the head plasma membrane activates hyaluronidase activity to facilitate the acrosome reaction and resulted in increment of fertilization rate. In the present study, analysis of the Na^+-K^+-ATPase was examined to certify its enzymatic activity for active transport. However there was no significant difference between two temperatures, the room and cold in those experimental animals. This result indicates that the active transport activity never be changed by the environmental temperature change at the level of the room and cold temperatures.

The scanning electron microscopic histochemistry obtained two photographs (Figs. 7a, b) and showed that there was no morphological difference on the head plasma membrane surfaces of the acrosome in the cold and room temperatures. This absence of difference indicates that the enzymatic secretion may persist even in the cold temperature during hibernation condition, although its quantitative analysis was not performed in the present study.

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<국문초록>

북부 온대지방에 서식하는 박쥐는 그 대부분이 동면을 하며, 그 생식유형은 특이하여 가을에 교미하여 사정된 정자는 자성생식도내에 긴 동면기간 동안 저장되어 있는 것으로 알려져 있다(예: 한국큰관박쥐). 환언하면 저장되어 있던 정자는 이듬해 봄에 배란되는 난자와 수정하여 초여름에 출산하게 된다. 본 연구는 월동하는 정자 두부 Plasma membrane의 성분과 활성 등 형태적 및 기능적 특성을 관찰 분석함으로써, 인공임신율을 높일 수 있는 보다 효과적인 한냉온도 정자저장방법을 모색하기 위하여 시도되었다. 동면하는 한국큰관박쥐와 햄스타 및 비동면동물(마우스)의 정자 Plasma membrane이, 실온과 한냉온도(박쥐-동면온도)에서, 생존율, 첨체반응율, 단백질분포, 능동수송에 관여되는 효소(Na^+-K^+-ATPase)의 활성 및 주사전자현미경적 조직화학상을 분석하였다.

본 연구에서 얻은 실험결과에 의거하면, 동면동물인 한국큰관박쥐와 햄스타 및 비동면동물인 마우스 정자의 생존율과 첨체반응율은, 실온과 한냉(박쥐-동면)온도간에 의의있는 차이를 발견할 수 없었고, 단백질 분포와 Na^+-K^+-ATPase 효소활성 및 주사전자현미경적 조직화학적 소견에 있어서도 두 온도간에 의의있는 차이는 없었다. 이와 같이 실온과 한냉온도 간에 형태적 기능적 차이가 없다고 하는 사실은, 동면동물이든 비동면동물이든 Spermatozoa plasma membrane이 동면중 또는 동면 이후에 안정적인 수정을 보장해 주는 형태적 및 기능적 보호구조임을 시사해 준다.

FIGURE LEGENDS

Fig. 2. Electron micrograph of intact spermatozoon of the bat.
Smooth outer plasma membrane, short acrosome and mitochondria of the middle piece are shown. A: acrosome, M: mitochondria. Scale bar=0.5 μm.

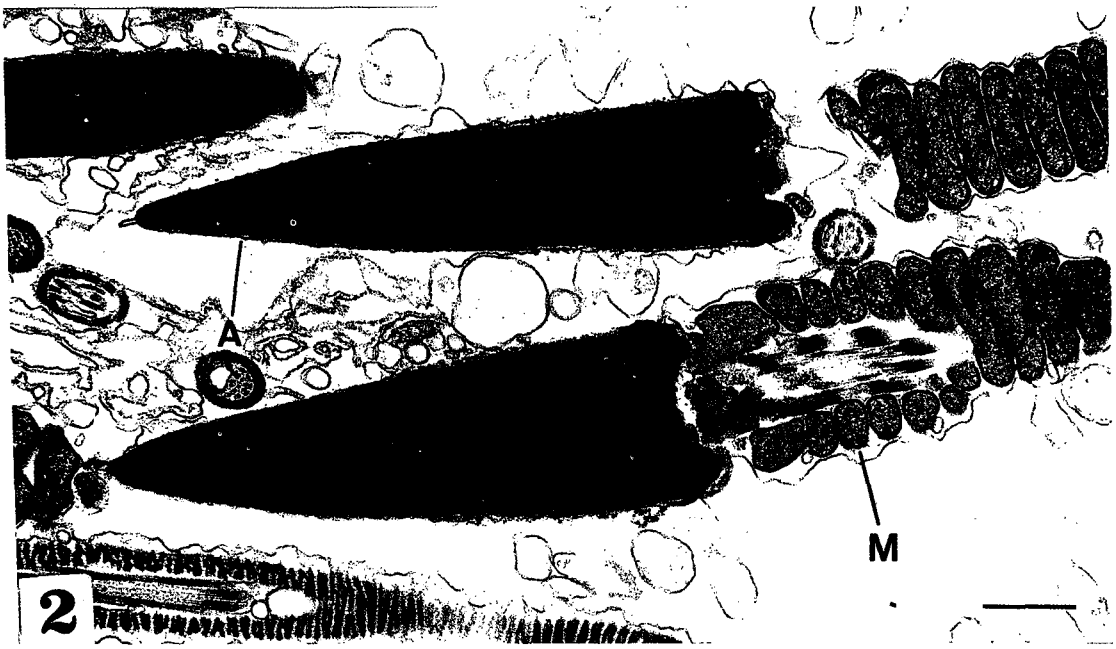
Fig. 3. Electron micrographs of sperm heads in the substrate-treated bat.
Na^+-K^+-ATPase-reacted substrate on the outer plasma membrane, reacted acrosome and mitochondria of the middle piece are evident at the room (a) and cold (b) temperatures. A: acrosome, S: reacted substrate, M: mitochondria. Scale bar=0.5 μm.

Fig. 4. Electron micrographs of sperm in the substrate-treated hamster.
Na^+-K^+-ATPase-reacted substrate on the outer plasma membrane, reacted acrosome and mitochondria of the middle piece are visible at the room (a) and cold (b) temperatures. A: acrosome, S: reacted substrate, M: mitochondria. Scale bar=0.5μm.

Fig. 5. Electron micrographs of spermatozoa in the substrate-treated mouse.
Na^+-K^+-ATPase-reacted substrate on the outer plasma membrane, reacted acrosome are shown at the room (a) and cold (b) temperatures. A: acrosome, S: reacted substrate, M: mitochondria. Scale bar=0.5 μm.

Fig. 6. Scanning electron micrograph of intact spermatozoa in the bat.
Long smooth head, neck and rough-surfaced middle piece are shown. H: head, M: mitochondria. Scale bar=1 μm.

Fig. 7. Scanning electron micrographs of spermatozoa in the substrate-treated bat.
Na^+-K^+-ATPase-reacted substrate on the plasma membrane at the room (a) and cold (b) temperatures are observed but no difference is found. H: head, S: reacted substrate. Scale bar=1 μm.



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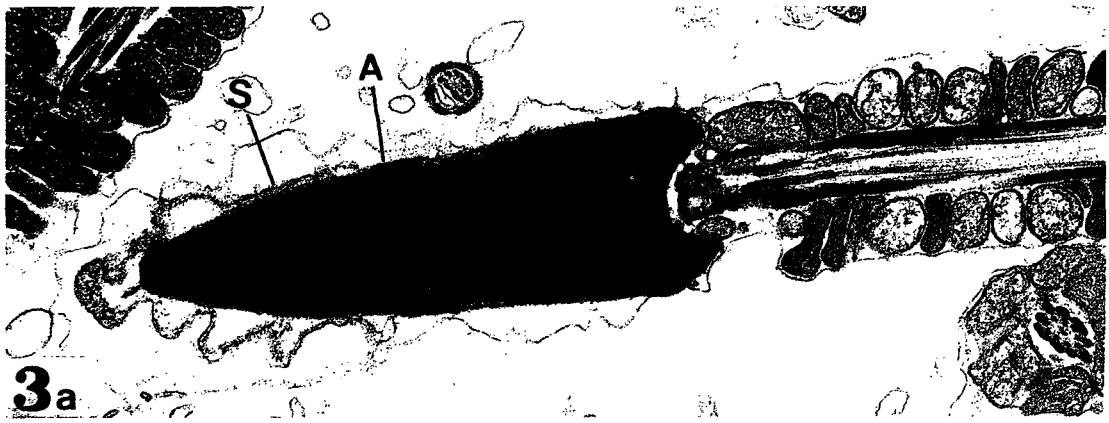

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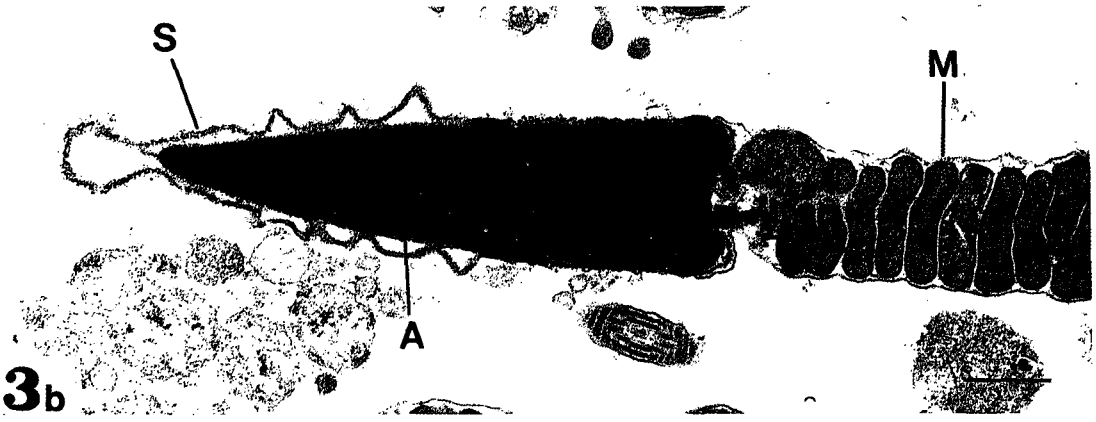

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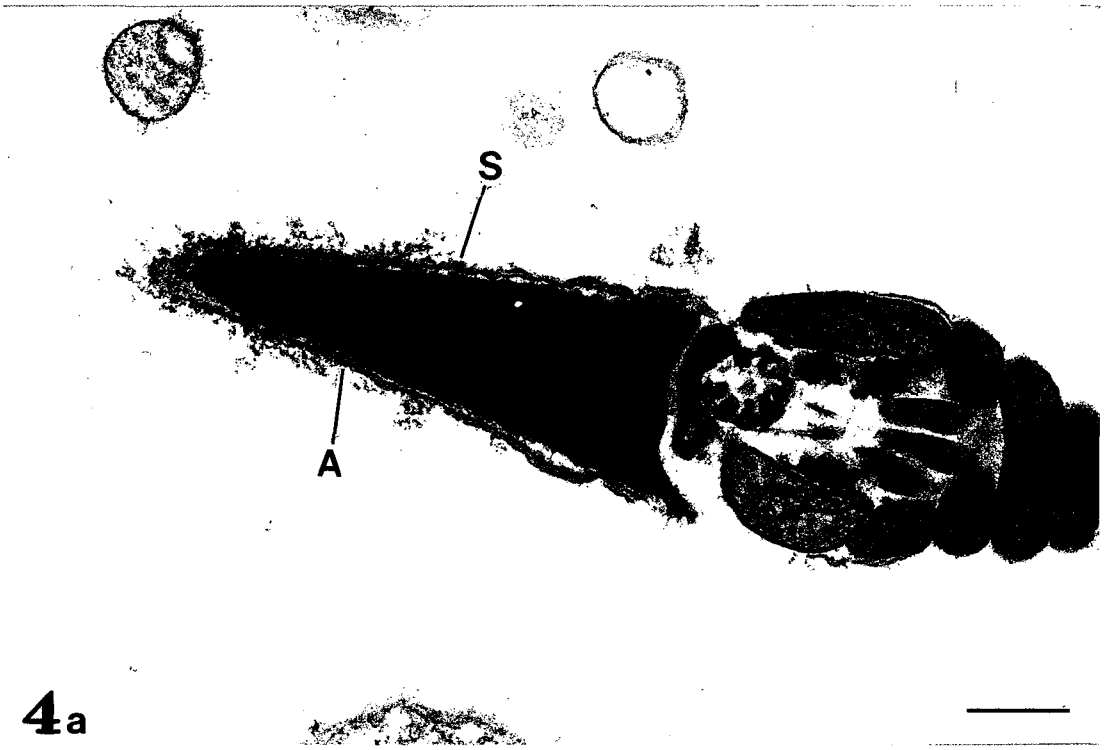

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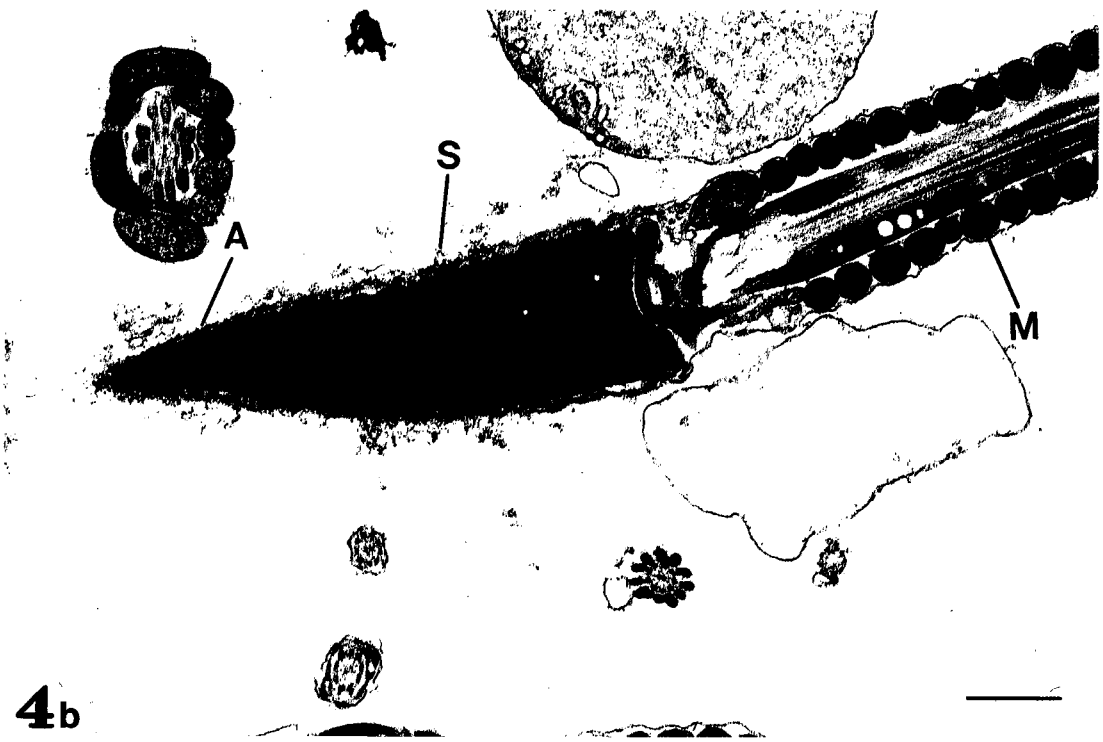

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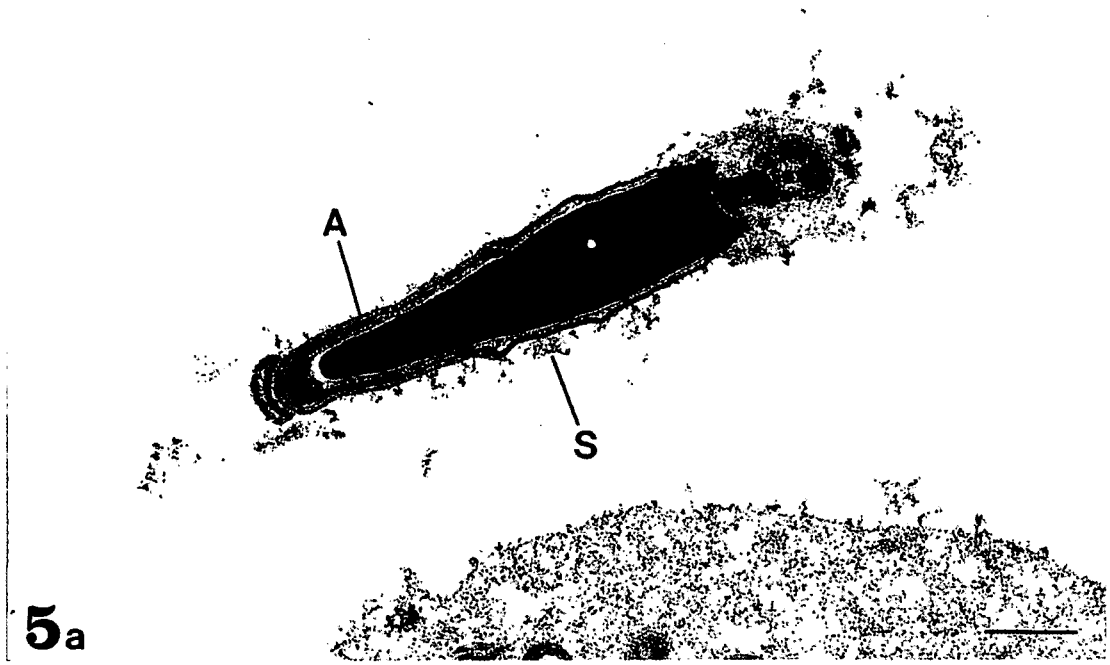

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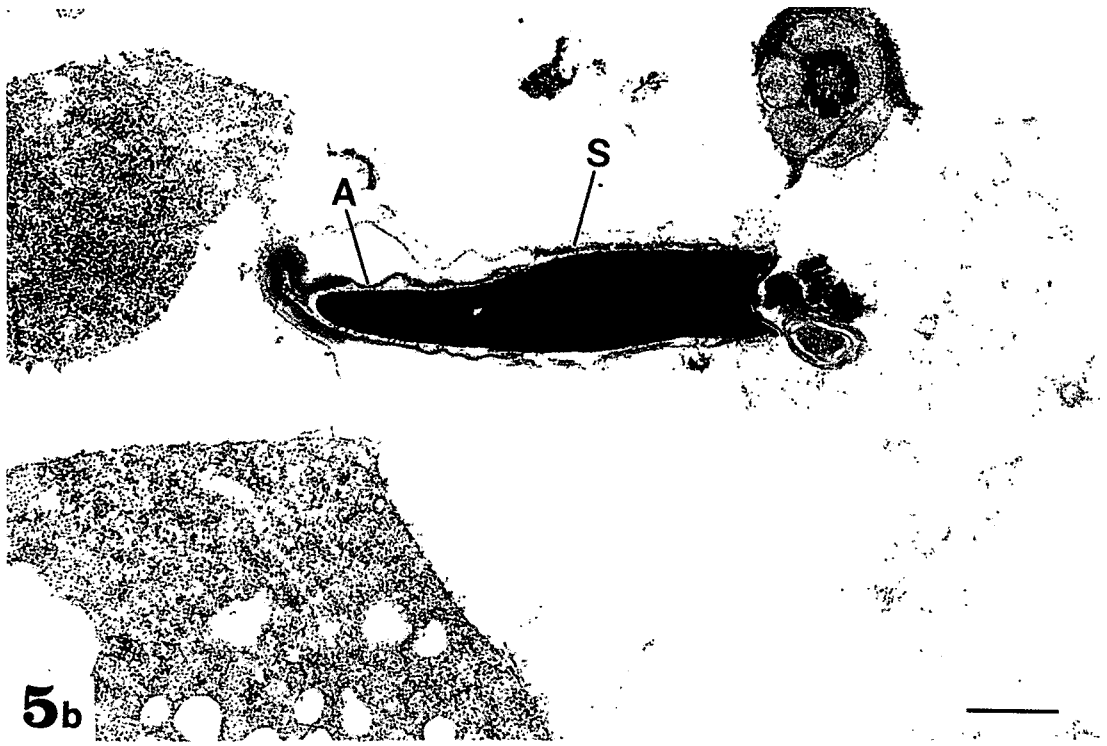

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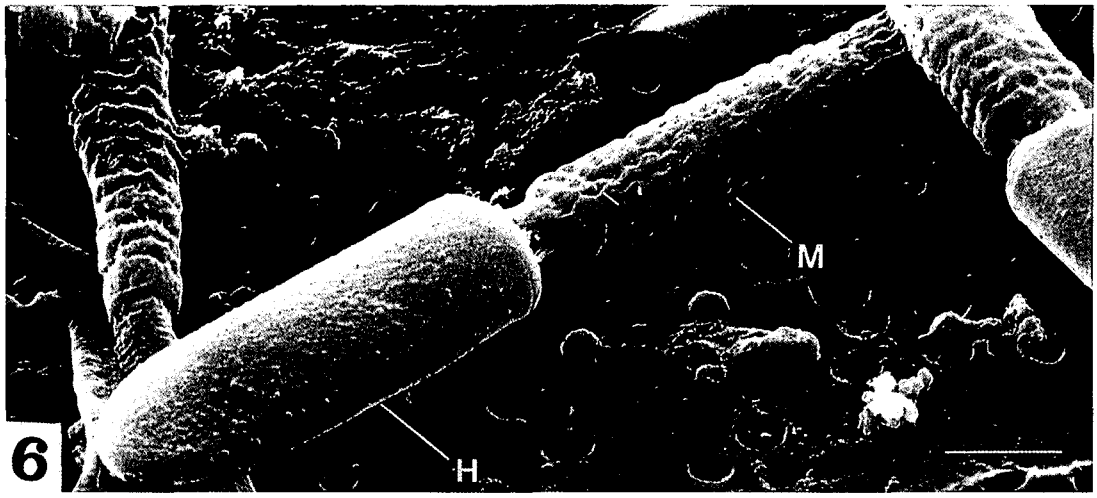
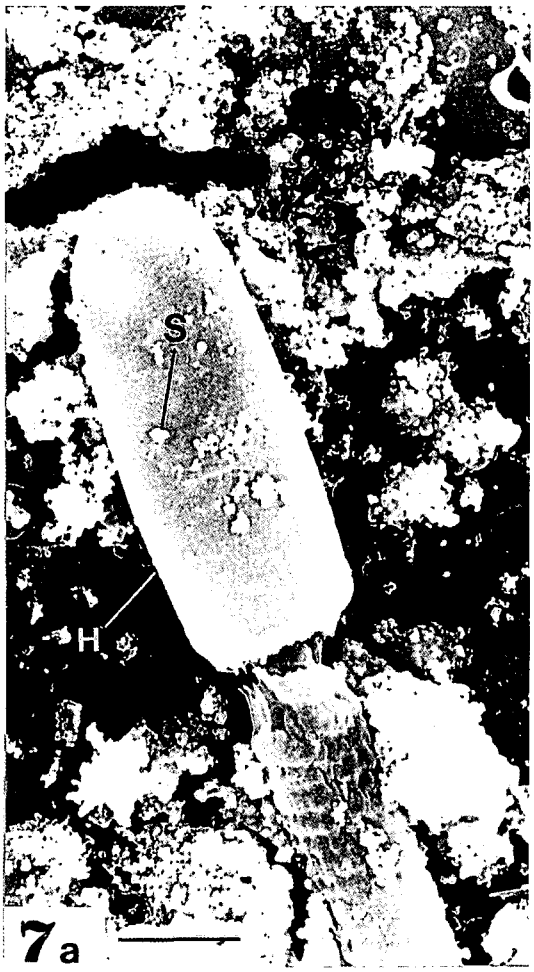

M
H
6


S
H
7a


S
S
7b