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A Comparative Study on Spermatozoan Maturation in the Hibernating Animals

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Abstract: Acrosome reaction usually occures just before fertilization in most mammals, and it has been known that Ca^{2+} plays an important role in the acrosome reaction and albumin also known as a critical factor for spermatozoan activities. The present study has been designed in order to observe maturing processes of the spermatozoa occurred in the ductus epididymidis and to clarify the relationships of Ca^{2+} concentrations with those processes, and to compare the enzymatic activities of ATPase and the lactate dehydrogenase of the spermatozoa in accordance with time before and after the spermatozoan maturation. From the results, we can confirm that most of the bat spermatozoa come to maturity within the epididymal cauda and may pass through capacitation outside the cauda. However it is expected to be studied that the fluctuation of spermatogenic activity depending on temperature changes and their relationships with the ductus epididymidis and their mutual influences.

Key Words: Acrosome reaction, Bat, Ca^{2+}, Epididymal cauda, Protein

INTRODUCTION

Several experimental models for long-term preservation of spermatozoa and ova through definite incubation period under the freezing or super-freezing temperatures have been proposed in order to elevate the fertilization rate[5]. In the Japanese horseshoe bat[12,14,18] and Korean horseshoe bat, there have been known that their fertilizing abilities were long sustained up to 4~6 months and ultimately their spermatozoa succeeded to be fertilized in the coming spring time when the ova ovulated. However, the mechanism of sustaining their capacitation has not yet been fully clarified although some experimental results have been proposed on the mouse[8,9] and hamster[5]. According to the above results, it is postulated that the female bats could maintain vital capacity of the spermatozoa introduced into their reproductive tracts through delaying capacitation[12] but this mechanism of maintenance has not yet been fully explained and discussed with a close connection to their accompanying results in accordance with falling of their body temperature[5,18].

It has been known in mammals that capacitation occurred during the maturation process

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of the epididymal spermatozoa and these changes took place in the nucleus, acrosome, and plasma membranes. It has been also known that the capacitation site within the ductus epididymidis differed from species to species: proximal cauda in the rats and hamsters[12,13,19]: only 50 % of spermatozoa found in the proximal cauda of the rabbits, where the capacitation occurred. Zhon *et al.* (1990) however has emphasized that the epididymal maturation of spermatozoa is necessary but not yet sufficient to reach the capacitation status for fertilization, and finally the spermatozoa have to pass through the acrosome reaction to be mature spermatozoa.

It seems likely that one of changes which can be occurred during spermatozoan maturation might include a compositional changes of the plasma membranes because conjugation of both the male and the female gamates occures essentially between two plasma membranes[2,7,8].

In the present study, three kinds of animals (mice, hamster, and bats) among many mammalian species were selected for experimental animals. Their related enzymatic activities and the intra-spermatozoan calcium ion concentrations, and the incidence rate of acrosome reaction were compared each other, with special regards to the data of bats and the those of another classical hibernation (hamster).

MATERIALS AND METHODS

Animal Breeding

In experimental group and bat was caught in Mt.Kueryong.

Preparation of the Spermatozoa

The spermatozoa were obtained from mice, hamsters and bats (the Korean greater horseshoe bats, *Rhinolophus ferrumequinum korai*) were captured in the Keumsung abandoned gold mine which is located in the Mt.Kueryong. The male Chinese hamsters (about 250 g in body weight) and the mice (25-30 g) were used in the experiment. The spermatozoa without albumin were treated with albumin.

Separation of the Caput and Cauda of the Ductus Epididymidis

After removing the fat tissue the ductus epididymidis, it was divided into 3 portions (caput, corpus, and caudal): the epididymal capsule was stripped out and only the caput and caudal portions were used for the materials to be measured.

Measurement of Acrosome Reaction Rate

The method of Talbot & Chacon (1981) was applied for observation of the acrosomes. The concentration of the spermatozoa was controlled at the range of 5×10^6-7×10^6/mL. The medium with spermatozoa and the same volume of Trypan Blue solution (albumin-free) were mixed, and the mixture was incubated in 37℃ for 15 minutes. After the 10 ml of phosphate buffered solution (PBS) was added to mixture and it was centrifuged at 600 xg for 8 minutes and then, the supernatant was removed and the sperm pellet added with 10 ml of PBS and re-centrifuged. After removing the supernatant, 1 ml of PBS was added. When the supernatant seems to be transparant or light blue in color, the mixture was added with 2 ml of glutaraldehyde and was fixed in room temperatrue for 20 minutes. After centrifuging, the supernatant was removed and the total mixture volume was controlled to 100 μl with distilled water. The mixture (10 μl) was dropped onto the slide glass and stained with Bismark brown solution at 40℃ for 5 minutes and washed with distilled water and then stained again with Rose bengal solution in room temperature for 27 minutes. After rinsing with distilled water, the specimen was dehydrated with graded alcohol (50, 75, 90, 100, 100 and 100 %) for each 5 minutes. The specimen was cleared with xylene for 5 minutes and mounted

and finally dried.

Measurement of $[Ca^{2+}]i$ of Spermatozoa

The sperm suspension was incubated at 37℃ for 45 minutes with 2 μmol/L Fura 2/AM in the dark room. The above suspension (2.5×10^6 cells/mL) was centrifuged in 6,000 rpm for 1 minute in order to remove extracellular Fura 2/AM leaked from the spermatozoa. The cell total volume within the cuvette was controlled to 2 ml and was measured by spectrofluorometer (340 nm excitation and fixed in 510 nm emission). The intracellular calcium was analysed by method of Grynkiewicz *et al.* (1985).

Measurement of Protein Concentrations of Testis

Protein was measured in testis of hamster and mouse in the control and expreimental group and bat in awake period (a period of non-reproduction and reproducion) and hibernation period. The analyzing method for protein was measured by modified Lowry method and a standard protein was used bovine serum albumin.

Measurement of Lactate Dehydrogenase Activity

Enzymatic activity of lactate dehydrogenase of spermatozoa has been measured in order to clarify whether the enzymatic activity could be a standard to discriminate maturity of spermatozoa in the caput of ductus epididymidis and the cauda of ductus epididymidis or not. The spermatozoa sampled from the testis were crushed by means of ultrasonic destruction and this suspension used as a source of mixtured solution were measured by the spectrophotometer (Hitachi, U-2000) in 340 nm in accordance with a definite time interval.

Measurement of Mg^{2+}-ATPase Activities

The measurement has been done by using the same enzymatic methods. After 100 μl of enzymes were added to the substrate (3 ml), the mixture was left in 30℃ for 10 minutes: the mixture including 6 ml of $MgCl_2$, 100 mM of NaCl, 20 mM of KCl, 40 mM of Tris-HCl, and buffered solution (pH 7.4). Then adding 50 μl of 0.1 M ATP solution (pH 7.4), the mixture was left in 30℃ for 4 minutes. Thereafter adding cold 0.1 ml trichloroacetic acid into the mixture and stopped the reaction to measure the free phosphorus level for the enzyme activities.

Statistics

The mean and 90 % confidence limits were calculated. We evaluated the statistical comparison by student's t-test and ANOVA test.

RESULTS

Measurement of $[Ca^{2+}]i$ of Spermatozoa

We designed experiments to evaluate $[Ca^{2+}]i$ in hamster, mouse and bat spermatozoa loaded with the intracellular calcium indicator, Fura 2/AM.

In ambient temperature, average $[Ca^{2+}]i$, measured 120 minutes after loading procedure, was significantly greater ($p<0.05$) in incubated sperm (181 ± 10.15 nmol/L, n=10 in hamster; 174 ± 5.5 nmol/L, n=10 in mouse: sperm prepared in medium containing bovine serum albumin) (Fig. 1).

In low temperature (10 ℃) after 14 weeks, average $[Ca^{2+}]i$, measured 120 minutes after loading procedure, was significantly greater in incubated sperm (156 ± 15.52 nmol/L, n=10 in hamster; 171 ± 8.33 nmol/L, n=10 in mouse) (Fig. 1).

There were no differences in $[Ca^{2+}]i$, between samples at ambient temperature and low temperature at ejaculated time and incubated time in hamster and mouse. In bat, $[Ca^{2+}]i$ reproductive period was significantly greater than that in non-reproductive period and hibernation

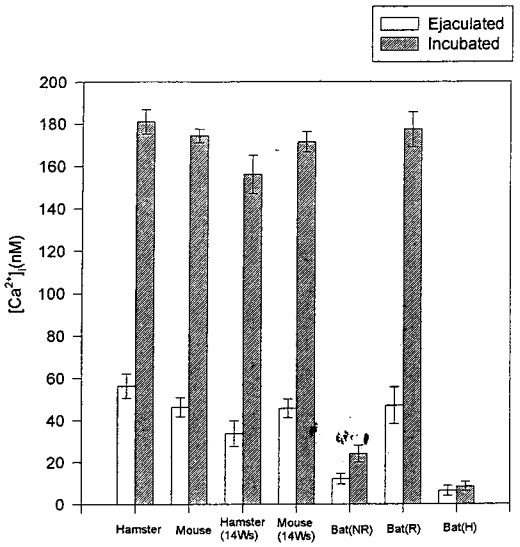

Fig. 1. Internal free calcium levels ($^{45}Ca^{2+}$) caudal epididymal spermatozoa loaded with 2 μmol/L Fura2/AM in the absence of external calcium. Each point represents the mean of five separates experiments; the vertical brackets represent the standard error of the mean (n=10). Ws=Weeks, NR=Non-reproduction, R=Reproduction, H=Hibernation.

period ($p<0.05$).

There were no differences in $[Ca^{2+}]i$, between samples non-reproductive period and hibernation period.

Measurement of Protein Content (μg/mL) of Testis

There were no significantly differences in protein of ambient temperature and low temperature group in hamster and mouse. But, in bat, there was statistically increased in reproductive period than other bat groups ($p<0.05$) (Fig. 2).

Acrosome Reaction Rate

At ambient temperature and low temperature group of hamster and mouse, although the percentage of living sperm decreased, there was repersented more high acrosome reaction rate than in incubated group with bovine serum albumin and in bat, increased acrosome reaction rate of incubated sperm in reproductive period

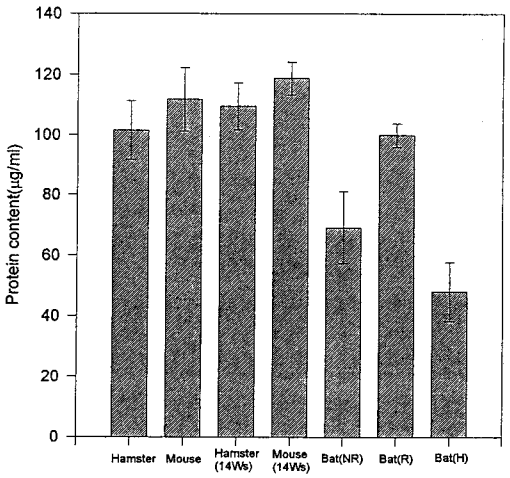

Fig. 2. Protein concentrations (μg/mL) of testes in hamster, mouse and bat. Each point the maen of five determination; the vertical brackets represent the standard error of the mean (n=10). Ws=Weeks, NR=Non-reproduction, R=Reprodduction, H=Hibernation.

(24.43 % versus 6.8 %) (Table 1). All sperm that died during acrosomal reaction exhibited a acrosomal breakdown leading to about 5-10 % increment of dead reacted sperm.

Measurement of Lactate Dehydrogenase Activity

The lactate dehydrogenase activity was measured in order to examine a possibility of the enzymatic activity to be a certain type of barometer to repersent maturity of the spermatozoa (Table 2). In mouse and hamster, there were no differences in enzymatic activity of ambient temperature and low temperature group. But, in bat, there was significantly differences in reproduction ($p<0.05$).

Based on the experimental results, in the all animals, the enzymatic activities of lactate dehydrogenase became higher, the nearer to the caudal portion difference of activities was not statistically significant. This suggests that only the lactate dehydrogenase itself could not be standard to discriminate the spermatozoan maturity.

Table 1. Acrosomal reaction rate in fresh and incubated hamster, mouse and bat spermatozoa. [a,b] $p<0.001$ (*t*-test), [c,d] $p<0.001$ (*t*-test)

	Sperm (Live)		
	Positive (%)	Negative (%)	Total (%)
T_a = 20 ~ 25°C			
Ejaculated (n=10)			
Hamster	5.77 ± 4.2 [a]	63.51 ± 12	68.87 [c]
Mouse	8.43 ± 6.78	75 ± 13	83.43
Incubated (n=10)			
Hamster	23.57 ± 21.4 [b]	27.77 ± 11	51.34 [d]
Mouse	29.23 ± 24.3	39.87 ± 17	69.1
T_a = 10°C, In the dark room			
After 14 weeks			
Ejaculated (n=10)			
Hamster	4.1 ± 3.5	47.27 ± 11	51.37
Mouse	7.43 ± 6.4	70.03 ± 10	77.46
Incubated (n=10)			
Hamster	10.23 ± 9.4	36.57 ± 12	46.8
Mouse	25.5 ± 21.3	40.37 ± 16	65.87
T_a = 10 ~ 15°C			
Bat: Reproductive Period			
Ejaculated (n=10)	6.8 ± 5.3	71 ± 10	77.8
Incubated (n=10)	24.43 ± 21	46.8 ± 17	70.23

Table 2. Comparisons of enzymatic activities in the hamster, mouse and bat. The results are expressed as the mean ± S.D. of more than four times determination. Student's *t*-test was used for statistical analysis of the data. N.S., Non-significant

Enzyme (Lactate Dehydrogenase)	Unit (μmole/min/mg protein)	p-value
T_a = 20 ~25°C		
Hamster	0.12 ± 0.05	N.S.
Mouse	0.13 ± 0.08	N.S
T_a = 10°C		
Hamster	0.11 ± 0.05	N.S.
Mouse	0.14 ± 0.10	N.S.
Bat		
Non-reproducive period	0.08 ± 0.04	
Reproductive period	0.14 ± 0.07	p<0.05
Hibernation period	0.04 ± 0.02	

Measurement of Mg^{2+}-ATPase Activities

Mg^{2+}-ATPase activities were measured to observe ATPase activities which is supplying the motility energy and it was confirmed that those enzymatic activities of the spermatozoa in the epididymal cauda were significantly high in the mice, hamsters, and the Korean gre-ater horseshoe bats (Table 3).

DISCUSSION

It has been known that Ca^{2+} infux in capacitation of animals[15,19,21], and the albumin has also been known to be a promoting factor to enhance capacitation of the mammalian spermatozoa[11]. In the hamsters[1], acrosome reaction did not occur in the absente condition of albumin. In experimental results of the present

Table 3. Comparisons of enzymatic activities in the hamster, mouse and bat. (Region: caude epididymal spermatozoa). The results are expressed as the mean ± S.D. of more than four times determination. Student's *t*-test was used for statistical analysis of the data. N.S., Non-significant

Enzyme (Mg^{2+}-ATPase)	Unit (μmole/min/mg protein)	p-value
T_a = 20 ~25°C		
Hamster	1.65 ± 0.75	p<0.05
Mouse	1.54 ± 0.65	p<0.05
T_a = 10°C		
Hamster	1.57 ± 0.66	p<0.05
Mouse	1.50 ± 0.54	p<0.05
Bat		
Non-reproducive period	1.00 ± 0.44	
Reproductive period	1.85 ± 0.85	p<0.05
Hibernation period	0.12 ± 0.10	

study using the hibernating mammals (Korean greater horseshoe bats, *Rhinolophus ferrumeguinum korai* and hamsters) and the non-hibernating mammals (mice), we have found the fact that the Ca^{2+} level of spermatozoa and the protein content have in certain extent been kept in proportional to the environmintal temperature in bat, In the mice and hamsters, a definite increment of Ca^{2+} level was observed in the ambient temperature and the low temperature incubation[5,10]. But in the bats, the significant differences in Ca^{2+} and protein level from those of the above results were found and which suggested that the mode of adaptation to the environmental might be quite different from the other mammals.

The present study has been designed in order to observe maturing processes of the spermatozoa occurred in the ductus epididymidis and to clarify the relationship of Ca^{2+} concentrations with those processes[14], and to compare the enzymatic activities of ATPase and the lactate dehydrogenase of the spermatozoa in accordance with time before and after the spermatozoan maturation[13,19].

In order to observe and confirm the acrosome reaction of the spermatozoa, the triple-staining method[16,17] was applied to the present study and we found that the acrosome reaction occurred more positively in the incubated spermatozoa than those immediately exudated from testes in bats like the other mammals as same as the experimental results of Talbot and Chacon (1980). However, on the contrary, it has been suggested by Talbot and Chacon (1981) that the incubation period should be seriously considered as a damaging factor for spermatozoa although the incubation could ingance the acrosome reaction rate.

Total survival number of bat spermatozoa during reproductive (coital) period did not fluctuate greatly and was different from those of other mammals (hamsters, mice). But no difference of the total survival rate was not observed in the low temperature in the hamster. This suggests that the adaptative capacity of bats to the low temperature has been once more demonstrated in the present study.

The intracellular concentrations of Ca^{2+} of spermatozoa were closely related to the acrosome reaction rate[2,7,8]: in the bat spermatozoa, the Ca^{2+} concentration was highly increased during the reproductive period although the total survival number of spermatozoa was not fluctuated greatly.

The protein content in testis was also proved to be closely related with the acrosome reaction and the total survival number of spermatozoa: According to the bat experimental results of Crichton *et al.* (1993), protein level to be

closely related with body temperature: during hibernation as the proteins and the carbohydrates play important roles in the basal metabolites for energy.

According to the experimental results of Meizel *et al.* (1983), it is shown that serotonin may be an inducing agent to acrosome reaction. For observing one of the maturation processes of spermatozoa, lactate dehydrogenase level was measured by Neilands (1955) method and significant differences of the enzyme activity were recognized in the reproductive (coital) period in the bats.

It is interesting to find a remarkable and significant of enzymatic activity (Mg^{2+}-ATPase) of spermatozoa of the epididymal cauda as shown by Alberts *et al.* (1983) indicating that this enzyme is dynein which has been shown to supply promoting energy for sperm motility. It is, therefore, possible to deduce from those results that spermatozoa have been matured in the epididymal cauda and obtained their vigorous motilities. A similar report has been demonstrated by Waites (1980).

Also, white (1989) reported that Ca^{2+} concentration of the mature spermatozoan cytoplasm being remarkably high and almost the same results were obtained in the present study for three species of animals. This data represents that the ejaculated spermatozoa of those animals passed through the capacitation in the epididymal cauda and then the mature spermatozoan volume became increased with special regards to observe the acrosome reaction rate by means of the triple staining technique, more acrosome reactions were found in the epididymal spermatozoa of the mice and the hamsters which have stayed in the definite period of time. On the contrary of the above observation, the spermatozoic activities of the bats were found to be active in the definite period of time, which has been known as a peculiar phenomenon of reproductive pattern.

The present study has been designed in order to observe maturing processes of the spermatozoa occurred in the ductus epididymidis and to clarify the relationship of Ca^{2+} concentrations with those processes, and to compare the enzymatic activities of ATPase and the lactate dehydrogenase of the spermatozoa in accordance with time before and after the spermatozoan maturation. From the above experimental results, the another can confirm that most of the bat spermatozoa come to maturity within the epididymal cauda and may pass through capacitation outside the cauda. However, it is expected to be studied that the fluctuation of spermatozonic activity depending on seasonal changes, and their relationship with the ductus epididymidis and their mutual influences.

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=국문초록=

동면동물의 정자성숙과정에 대한 비교 연구

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한림대학교 의과대학 생리학교실**

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대부분의 포유동물들에서 수정전에 정자의 첨체반응이 일어난다고 알려져 있으며 첨체반응에서 Ca^{2+} 이 중요한 역할을 한다고 알려져 있다. 단백질 또한 정자의 생리적 역할에 중요한 요소로 작용한다고 보고되고 있다. 본 실험에서는 동면동물의 부정소에서 정자성숙과정을 비교 관찰하고 성숙과정에 있어 Ca^{2+} 농도와의 연관성을 규명하고 정자성숙 전, 후에 정자성숙에 영향을 미치는 Mg^{2+}-ATPase, lactate dehydrogenase의 작용을 비교, 분석하였다. 그 결과 다음과 같은 결과를 얻었다. 대표적 동면동물인 박쥐의 경우 활동기에 다른 동면동물인 햄스터, 항온동물인 생쥐와 마찬가지로 부정소미에서 정자성숙과정을 거쳤으며 교미시 대부분의 정자는 부정소미에서 더욱 활발한 운동성을 보였으며 부정소미 밖에서 수정능획득 과정을 거치는 것으로 관찰되었다. 그러나 동면동물의 환경조건 특히 온도의 변화가 부정소에서의 정자성숙과정에 영향을 주는지에 대해서는 더 자세한 연구가 필요하다고 사료된다.

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