UPTAKE OF α-AMINOISOBUTYRIC ACID (AIB) BY ROOSTER SPERMATOZOA

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Summary

This experiment was designed to determine whether α-aminoisobutyric acid (AIB) can be used to predict membrane function of spermatozoa by measuring the uptake of AIB by fresh, stored and frozen-thawed rooster spermatozoa. When spermatozoa were stored at low temperature (0~3°C) for 24 h, no difference was found in AIB uptake compared with fresh spermatozoa, whereas storage for 48 h resulted in a slight increase in AIB uptake by spermatozoa. On the one hand, the uptake of AIB by frozen-thawed spermatozoa was less than that by fresh spermatozoa. This suggests possibility of a different membrane transport system between spermatozoa preserved at low temperature (0~3°C) and those frozen-thawed. Glycerol used as cryoprotectant may modify rooster sperm membrane in a different manner from cold preservation. Ouabaine (10^{-4} M) caused a slight decrease in AIB uptake, but caffeine (10^{-2} M) did not influence spermatozoal AIB uptake. These results indicate a successful application of AIB to rooster spermatozoa as a mean for measuring sperm membrane function and suggest a possible alteration of membrane transport system in rooster spermatozoa between cold (0~3°C) and cryopreservation (196°C).

(Key Words: Rooster, Spermatozoa, Preservation, Membrane, AIB Uptake, Freeze-Thaw)

Introduction

The decline in fertility with *in vitro* storage of rooster spermatozoa has been considered to be, in part, due to damage to the plasma membrane of spermatozoa. Brown et al. (1971), Buckland (1971a, b) and Bernon and Buckland (1975) have reported enzyme leakage from spermatozoa after in vitro storage and suggested that the degree of enzyme leaking could be an indicator of sperm cell damage. However, a more direct method of measuring sperm membrane function would be useful in assessing damage due to handling and in vitro storage technique. Recently, the determination of the uptake of α-aminoisobutyric acid (AIB), non-metabolizable amino acid (Christensen et al., 1956; Akedo and Christensen, 1962), by living cells has been one of the most useful tool for measuring cell membrane transport.

This experiment was performed to determine whether AIB can be used to predict membrane function of spermatozoa by measuring the uptake of AIB by fresh, stored and frozen-thawed rooster spermatozoa.

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Received December 21, 1989
Accepted March 22, 1990

Materials and Methods

Semen preparation

Male fowls were White Leghorn commercial strain from Fukuoka Prefectural Livestock Station and they were housed individually in wire cages and given food and water *ad libitum*, and subjected to a daily photoperiod of 14 h light/10 h dark. The lumbo-sacral massage method described by Bogdonoff and Schaffner (1954) was applied to obtain semen. Semen samples were collected from at least 6 males on a two-times weekly routine. Collected semen were pooled immediately after collection and divided equally into three aliquots; one for fresh semen, and the remaining for storing and freezing semen, respectively. Spermatozoa for fresh semen were washed twice with two volumes of iced-buffer (Goldfine et al., 1972) containing 11mM of glucose, and spermatozoal concentration was adjusted to 1.0 to 3.0 x 10^9 sperm cells/ml. To determine the uptake of AIB by stored spermatozoa, semen were diluted three times (1:3) with Lake's non-glycerolizing fluid (Lake and Stewart, 1978), and preserved for 24 h and 48 h in a refrigerator (0~3°C). For freezing spermatozoa, semen samples were diluted with Lake's glycerolizing solution (Lake and Stewart, 1978) and frozen by employing the method described by Mitchell et al. (1975) and Forgrave and Baker (1975).

Ouabaine (10^{-4} M), the specific inhibitor of $Na^+ - K^+$ – ATPase (Skou, 1965), and caffeine (10^{-2} M), a cyclic nucleotide phosphodiesterase inhibitor (Robinson et al., 1971), were supplemented to sperm suspension so as to examine the effects of the agents on the uptake of AIB by rooster spermatozoa.

Determination of AIB uptake

Following cold preservation and freezing, spermatozoa were separated by centrifugation at 600 x g for 10 min at 3~5°C, and resuspended with iced-buffer before adjusting sperm density to 1.0 to 3.0 x 10^9 sperm cells/ml. For all semen samples from fresh, stored and frozen spermatozoa, aliquots (1.0 ml) of sperm suspension were placed into small test tube with rubber top and then preincubated in a shaking water bath at 37.5°C for 10 min. Immediately after preincubation, the α-amino (methyl-^{3}H) isobutyric acid (45 μCi/μM) was added to sperm suspension. The number of spermatozoa was again determined and this number was used for all calculation thereafter. Duplicate 100 μl aliquots of sperm suspension were taken out at intervals of 0, 30, 60 and 120 min of incubation. The aliquots (100 μl) were centrifuged and the spermatozoal pellets were washed according to the method of Goldfine et al. (1972). The supernatants were discarded and 100 μl of 10% perchloric acid were added to each of spermatozoal pellet which was then transferred to a counting vial with the perchloric acid and counted with a Beckman LS-235 liquid scintillation counter. Initial uptake of AIB, referring to as interval of 0 min after incubation, was expressed as an amount of AIB taken up by spermatozoa immediately after the addition of labelled amino acid. To determine the rates of AIB uptake, pM of AIB taken up by 10^8 sperm cells per min was calculated by the slope of plot of AIB uptake versus incubation time (min).

Results have been expressed as picomoles of AIB taken up per 10^8 sperm cells. The results of these experiments were analysed for statistical significance using analysis of variance followed by *t*-test for comparing differences between the groups (Steel and Torrie, 1980).

Chemicals

α-amino (methyl-^{3}H) isobutyric acid (10 Ci/mM) was purchased from the Nuclear England, London. All other reagents were of the best grade commercially available and were obtained from Sigma (St. Louis, MO).

Results

A preliminary examination showed that a concentration of 30 μM of AIB, a sperm density of between 1.0 and 3.0 x 10^9 sperm cells/ml and 45 μCi/μM of [^{3}H-AIB] in the incubation media would be suitable for this experiment (Fujihara et al., 1983; Fujihara and Koga, 1986).

The uptake of AIB by fresh and stored spermatoza

Figure 1 depicts that rooster spermatozoa preserved at 0 ~ 3°C for 48 h accumulated more AIB than fresh and 24 h-stored spermatozoa, but the rates of AIB uptake had a tendency to increase

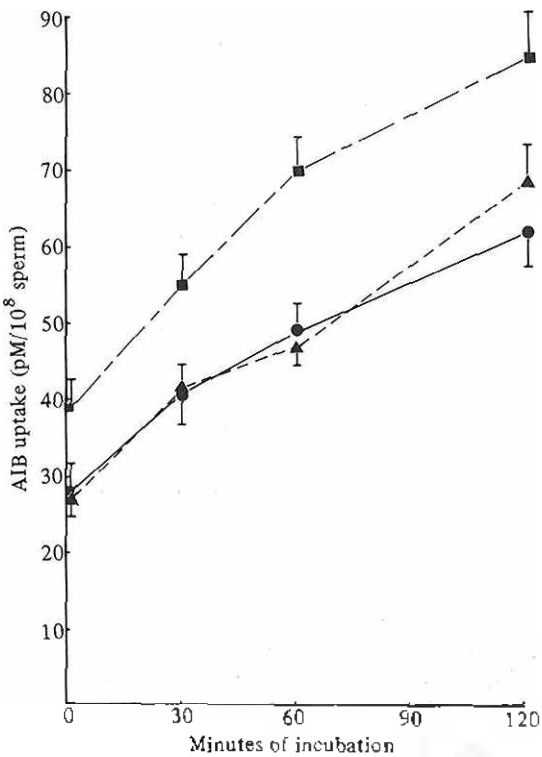

Figure 1. Time course of AIB uptake by fresh and stored rooster spermatozoa. ●—●, fresh; ▲—▲, 24 h-stored; ■—■ 48 h-stored spermatozoa. Vertical bar indicates S.D.

TABLE 1. INITIAL UPTAKE AND UPTAKE RATE OF AIB BY ROOSTER SPERMATOZOA

Treatment		Initial uptake (AIB pM/10^8 sperm)	Uptake rate (AIB pM/10^8 sperm/min)
Stored	Control	28.34 ± 11.08[a]	0.27 + 0.06
	24 hr	28.16 ± 7.72	0.34 ± 0.11
	48 hr	39.72 ± 12.74*	0.37 ± 0.16
Frozen	Control	34.89 ± 9.88	0.35 ± 0.06
	Frozen	28.74 ± 10.96	0.29 ± 0.16
Ouabaine (10^{-4} M)	Control	38.06 ± 4.68	0.23 ± 0.07
	Ouabaine	30.91 ± 8.15	0.23 ± 0.04
Caffeine (10^{-2} M)	Control	27.13 ± 8.22	0.28 ± 0.04
	Caffeine	26.51 ± 4.57	0.33 ± 0.27

[a]Means ± S.D.
*$p < 0.05$ vs. control

with increasing time of preservation though no significant difference was found among the three groups. However, initial uptake of AIB by 48-stored spermatozoa was significantly ($p < 0.05$) higher than that by fresh and 24 h-preserved spermatozoa (table 1). The values of initial uptake were nearly the same for both fresh and 24 h-stored spermatozoa (figure 1 and table 1).

AIB uptake by frozen-thawed spermatozoa

AIB accumulation in frozen-thawed spermatozoa was less than that in fresh spermatozoa (figure 2). Although initial uptake and uptake rate slightly tended to decrease when spermatozoa were frozen and thawed, no significant difference ($p > 0.05$) was observed in both initial uptake and the rate of uptake between fresh and frozen-thawed spermatozoa (figure 2).

Effects of ouabaine and caffeine on spermatozoal AIB uptake

Addition of ouabaine (10^{-4} M) to sperm suspension slightly reduced the uptake of AIB by spermatozoa (figure 3), but there was no statistical significance in both the rate of uptake and initial uptake between the two semen groups. On the other hand, AIB uptake by spermatozoa was not influenced significantly by the presence of caffeine at the concentration of 10^{-2} M (figure 4).

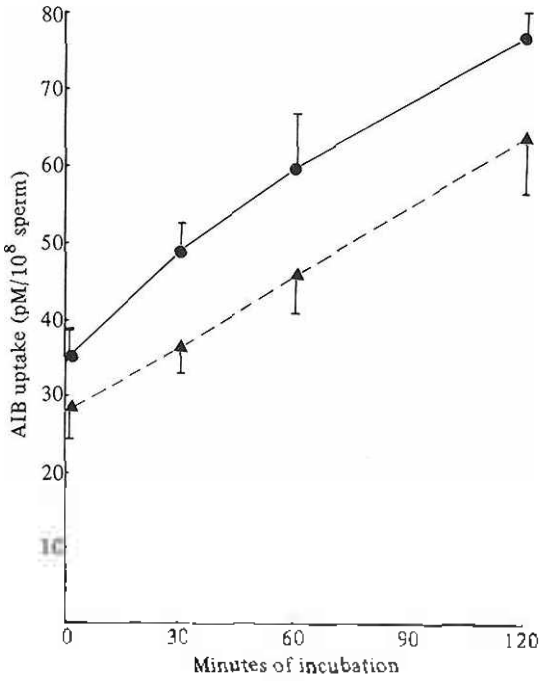

Figure 2. Time course of AIB uptake by fresh and frozen-thawed rooster spermatozoa.
●—●, fresh; ▲---▲, frozen-thawed spermatozoa.
Vertical bar indicates S.D.

Discussion

Preservation of rooster spermatozoa at 0-3°C for 24 h lead to an accumulation of the same amount of AIB as that of fresh spermatozoa, but

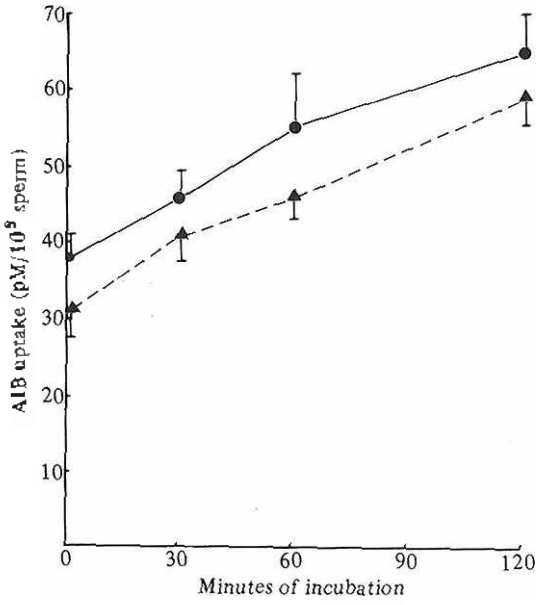

Figure 3. Effect of ouabaine(10^{-4}M) on the time course of AIB uptake by fresh rooster spermatozoa.
●—●, fresh; ▲---▲, ouabaine-treated spermatozoa.
Vertical bar indicates S.D.

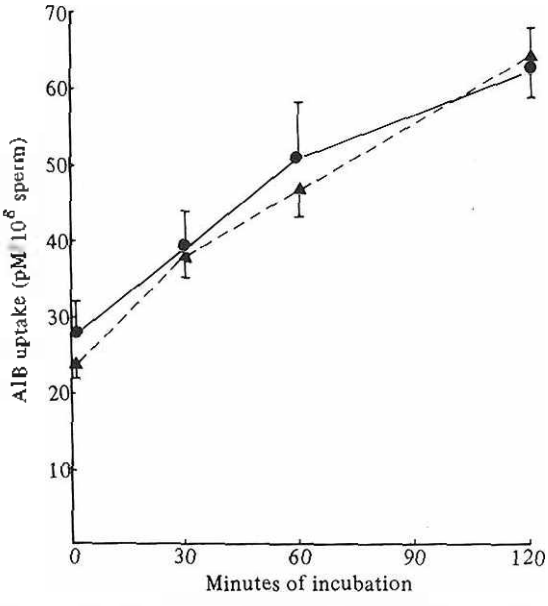

Figure 4. Effect of caffeine (10^{-2} M) on the time course of AIB uptake by fresh rooster spermatozoa.
●—●, fresh; ▲---▲, caffeine-treated spermatozoa.
Vertical bar indicates S.D.

the rate of AIB uptake was shown to increase slightly when spermatozoa were stored for one day. On the other hand, 48 h storage of spermatozoa significantly ($p < 0.05$) enhanced initial uptake of AIB, but no difference was found in the rate of uptake between 48 h- and 24 h-stored or fresh spermatozoa (table 1 and figure 3). A significant difference ($p < 0.05$) in initial uptake of labelled amino acid between 48 h- and 24 h-stored or fresh spermatozoa suggests that a certain kind of modification might have occurred in spermatozoal membrane with increasing period of storage at low temperature ($0 \sim 3^{\circ}C$). Prolonged period of in vitro preservation of fowl spermatozoa is, therefore, likely to induce some sort of changes in membrane permeability of sperm cells.

Frozen-thawed rooster spermatozoa accumulated less amount of AIB than fresh spermatozoa, suggesting that preservation of spermatozoa in liquid nitrogen ($-195^{\circ}C$) seemed to influence membrane transport system of spermatozoa in a different manner from low temperature storage ($0 \sim 3^{\circ}C$). This infers that a different mechanism may exist in the uptake of labelled amino acid between cold stored and frozen spermatozoa. It is, therefore, likely that glycerol contained in semen extender for freezing semen may modify membrane function of rooster spermatozoa as well as being a better cryoprotective agent. Cryoprotectants such as glycerol have been considered to alter membrane permeability of living cells (Meryman, 1971). It has also been reported that the rate of uptake of amino acid was reduced considerably by glycerol addition to frozen *Neurospora crassa* (Wellman and Pendyala, 1979). In this experiment, glycerol probably modified membrane permeability of fowl sperm cells without affecting survival of spermatozoa.

In the present study, on the one hand, ouabaine at 10^{-4} M slightly suppressed AIB uptake by spermatozoa, indicating that the uptake of AIB by rooster spermatozoa may partly be energy-dependent. After 2 h of incubation at $37.5^{\circ}C$, the accumulation of AIB in ouabaine-treated spermatozoa was still less than that in fresh spermatozoa, suggesting a possibility that rooster spermatozoa can retain their membrane undamaged even after considerably prolonged time of incubation.

Ouabaine has been demonstrated to have an inhibiting action on AIB transport into living cells

(Tcuabi and Jeanrenaud, 1969; Goldfine et al., 1972; Iwamoto and Williams, 1980; Keryer and Rossignol, 1980).

Caffeine at the concentration of 10^{-2} M did not have any influence on rooster spermatozoal AIB uptake. Caffeine has generally been accepted to stimulate sperm motility in a fashion of producing cAMP in the cells treated. The results of the present study suggest that intracellular activation of metabolism, including enhancement of cAMP level in sperm cells, may not be involved directly in the uptake of labelled amino acid. It has also been shown that theophylline, considered to be a cyclic nucleotide phosphodiesterase inhibitor similar in function to caffeine, had no effect on amino acid trasport into living cells (Tews et al., 1970; Schwartz, 1974; Pariza et al., 1976, 1977). Thus, cAMP may not participate in enhancement of the uptake of AIB by rooster spermatozoa. However, there are some discrepancies regarding the effect of cAMP-producing agents and of addition of these substances on AIB transport into living cells, depending upon the kinds of cells and of chemicals administered (Guidotti et al., 1978).

Acknowledgements

The authors wish to thank Mr. N. Yoshihiro for his skilled technical assistance throughout the present study. This study was supported by a Grant-in Aid for Scientific Research (No. 60560285) from the Ministry of Education, Science and Culture, Japan.

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