RESEARCH ARTICLES

EFFECTS OF ION CONTENT AND PH OF MEDIUM ON MOTILITY OF TILAPIA (*OREOCHROMIS NILOTICUS*) SPERM

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(Received May 1, 1997)

ABSTRACT

The motility of tilapia sperm was found to vary depending on the ionic composition and pH of the diluting solutions. Artificial freshwater at pH 7.0 was the best medium for maintaining both the duration and capacity of sperm motility. Concentration of Na^+ (1.5 mM), K^+ (0.5 mM), Ca^{2+} (1.0 mM) and Mg^{2+} (0.5 mM), the same as those of artificial freshwater, activated the motility maximally. Permeabilized tilapia sperm lost functional motility which was maximally restored in the presence of 1 mM ATP. Ouabain, sodium vanadate and 1-fluoro-2,4-dinitrobenzene (FDNB) were shown to inhibit the flagellar beating with $K_{0.5}$ values of 1.3 mM, 25 μM and 1.6 μM, respectively. Conversely, 1-methyl-3-isobutylxanthine activated flagellar beating with $K_{0.5}$ of 0.24 mM. Inhibition of Na^+/K^+-ATPase activity by ouabain, inhibition of dynein ATPase activity by sodium vanadate, inhibition of creatine kinase activity by FDNB and inhibition of phosphodiesterase activity by 1-methyl-3-isobutyl-xanthine were found to have $K_{0.5}$ values of 1.6 mM, 25 μM, 1.8 μM and 0.20 mM, respectively. These values were close to those for the inhibition of the flagellar beating. Thus, an involvement of Na^+/K^+-ATPase, dynein ATPase, creatine kinase and phosphodiesterase in the motility of tilapia sperm is revealed.

INTRODUCTION

Studies on motility of fish sperm have been restricted to a very limited numbers of almost 20 species whereas abundant species of fish are known. Tilapia is one of commercially important species in Thailand. Only limited information is available on its sperm motility. A better understanding of the biology of tilapia sperm will be useful for management in fish farming. This species has external fertilization and gametes are exposed at least for a short period of time to the external medium. The motility of the spermatozoa of freshwater fish following spawning is usually of short duration (<30 sec to 3 min)[20], the extent varying according to the pH and ionic composition of the diluting medium.[2,4,5,12] An increase in the duration of sperm motility appears to correlate with an increased capacity of the sperm to fertilize an ovum[41] and any increase in Na^+ or K^+ concentration which inhibit sperm motility will impair fertilization.[1,19] Sperm motility is maintained by ATP synthesized by mitochondrial respiration. The ATP is hydrolysed primarily by the dynein ATPase present in the flagellar axoneme[16] and *in vitro* motility is inhibited by sodium vanadate, a dynein ATPase inhibitor.[11] Motility is also impaired when creatine kinase activity is inhibited with 1-fluoro-2,4-

dinitrobenzene, presumably due to inhibition of energy transport between mitochondria and tail of sperm mediated by phosphocreatine shuttle and creatine kinase action.[35,36]

An involvement of the nucleotides or cAMP in the control of sperm motility is well known.[14,28] Sperm of many species possess phosphodiesterase activity and either exogenously added cyclic nucleotides or an increase in the intracellular cAMP can initiate sperm motility.[14,29] Papaverine and caffeine, inhibitors of phosphodiesterase, markedly increase motility of bovine epididymal sperm and cause marked decreases in sperm ATP concentration.

Sperm are easily permeabilized by treating them with a non-ionic detergent such as Triton X-100 and permeabilized sperm model has been used extensively in studies of dynein ATPase[17] and useful data as the effects of ATP, cAMP and pH on rat epididymal sperm has been gained by this mean.[38]

The aim of the present paper is to evaluate any factors affecting motility of tilapia sperm. In this study of tilapia sperm, investigations were made on : (1) the effect of ion content and pH of medium on sperm motility, (2) the role of ATP on flagellar beating, and (3) the involvement of dynein ATPase, Na^+/K^+-ATPase, creatine kinase and phosphodiesterase in sperm motility.

MATERIALS AND METHODS

Chemicals

Sodium vanadate, 1-fluoro-2,4-dinitrobenzene (FDNB), 1-methyl-3-isobutylxanthine (MIX), 5'-adenosine triphosphate (ATP), dithiothreitol (DTT), ethylenediamine tetraacetic acid (EDTA) and all common chemicals were purchased from Sigma (Uppsala, Sweden).

Preparation of sperm

Adult male tilapia were anaesthetized with 5 ppm quinaldrin. The testes were surgically removed and kept at 0°C not more than 10 min before being used. Sperm were freshly extruded dry by puncturing the testes with a hypodermic needle and used immediately.

Test solutions

Test solutions used in experiments of sperm motility were single distilled water, artificial freshwater and filtered freshwater. Artificial freshwater was distilled water containing 10 mM Tris-HCl, 1.5 mM NaCl, 0.5 mM KCl, 1.0 mM $CaCl_2$ and 0.5 mM $MgCl_2$ with pH 7.0.[40] Filtered freshwater was collected from natural pools, filtered through a Whatman filter paper number 3 and kept at 4°C prior to being used. Its pH was approximately 7.0.

Sperm motility

Sperm extruded from testes (5 μl) were diluted with a test solution to a final volume of 1 ml to form a uniform suspension. Ten microlitres of the sperm suspension was quickly placed in a haemocytometer and sperm movement assessed under a phase-contrast microscope (40X objective).[8] The first measurement was taken 30 sec after dilution. A total of two hundred sperm in a sample were observed and an estimate of the percentage of sperm showing progressive motility obtained. All procedures were carried out at room temperature (25-28°C).

Flagellar beating assay

Permeabilized sperm were prepared according to the method of Treetipsatit and Chulavatnatol (38). A small aliquot of the sperm suspension (0.1ml) containing 10^5 sperm was

treated for 30 sec with 1 ml of the permeabilizing solution, containing 0.1% Triton X-100 in 10 mM Tris-HCl, pH 7.0 and then reactivated with ATP at a specified concentration. After a predetermined time, 10 μl of the mixture was placed in a haemocytometer. A differential count of 200 sperm models was made to identify those with beating and those with non-beating flagella under a dark-field microscope. The counting was usually completed within 5 min after withdrawal of the sample. All steps were carried out at room temperature. The percentage of flagellar beating was calculated from the number of beating sperm X (100/200)%.

Enzyme assays

Sperm were washed twice with artificial freshwater, pH 7.0, by centrifugation at 2,800 g for 10 min at 4°C. The packed cells were resuspended in 50 mM Tris-HCl, pH 7.5 containing 0.05 % Triton X-100, 0.1 M NaCl, 10 mM KCl and 5 mM $MgCl_2$. The suspension was then homogenized by 10 strokes in a Dounce homogenizer and the homogenate assayed for enzyme activity. All enzyme activity except phosphodiesterase was determined using a pyruvate kinase-lactate dehydrogenase linked system in which hydrolysis of ATP is coupled to the oxidation of NADH. The reaction was initiated by addition of NADH and $O.D._{340}$ was measured. Ouabain-sensitive Na^+/K^+-ATPase was assayed by the method of Gache *et al.*[13] The final reaction mixture (1 ml) contained 0.14 mM NADH, 4 mM phosphoenolpyruvate, 2 mM ATP, pyruvate kinase 7.5 units, lactate dehydrogenase 10 units, 0.1 M NaCl, 10 mM KCl and 5 mM $MgCl_2$ in 50 mM Tris-HCl, pH 7.5. The inhibition of Na^+/K^+-ATPase activity was performed in the presence of various concentrations of ouabain. Dynein ATPase was determined following the procedure of Gibbons *et al.*[15] The reaction mixture (1 ml) contained 0.1 mM NADH, 1.5 mM phosphoenolpyruvate, 0.5 mM ATP, pyruvate kinase 16 units, lactate dehydrogenase 22 units, 0.5 mM EDTA, 150 mM KCl, 5 mM $MgCl_2$ and 1 mM DTT in 50 mM Tris-HCl, pH 8.0. The inhibition of dynein ATPase activity was determined in the presence of various concentrations of sodium vanadate. Creatine kinase was estimated by the method of Tombes and Shapiro.[36] The assay was performed in the same reaction mixture of Na^+/K^+- ATPase with the addition of 50 mM creatine in the presence of various concentrations of 1-fluoro-2,4-dinitrobenzene. Phosphodiesterase was determined by the method of Utarabhand[39] in a 100-μl volume containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM cAMP and 0.2 μCi ^{3}H-cAMP (36.1 Ci/mmol) in the presence of various concentrations of 1-methyl-3-isobutylxanthine. After incubation for 10 min at 30°C, the reaction was terminated by boiling for 2 min. Samples were cooled and then 25 μg of *Crotalus atrox* snake venom was added, incubated for 10 min at 30°C, and placed on ice. Labeled adenosine was separated by AG 1X2 ion-exchange column chromatography. An aliquot of the filtrate was counted in 25% Triton X-114-xylene based cocktail.

RESULTS

When extruded from the tilapia testes, all sperm were non-motile. Upon dilution with a test solution, a uniform suspension of sperm was detected and the sperm immediately began vigorous movement with the highest percent motility (73%) being observed upon dilution in artificial freshwater, pH 7.0 (Table 1). Filtered freshwater gave less (43%) and distilled water the poorest motility (33%). Sperm progressively lost their motility after dilution in the test solutions (Fig.1). Among the 3 test solutions, duration of sperm motility was maintained longest in artificial freshwater (6 min) compared with 3 and 2 min in filtered freshwater or distilled water, respectively.

When the artificial freshwater buffered with 10 mM sodium acetate to pH 5 and 6or with 10 mM Tris-HCl to pH 7 to 9 were tested, maximal motility was observed at pH 7.0 (Fig.2).

As artificial freshwater consistently gave good initiation and prolongation of motility, the effects of changing its ionic composition were studied. Among the ions studied, all of them were activators for sperm motility. The optimal concentration of $MgCl_2$, KCl, NaCl and $CaCl_2$ when dissolved in 10 mM Tris-HCl, pH 7.0 were 0.5 mM, 0.5 mM, 1.5 mM and 1.0 mM, respectivety (Fig.3).

Upon treatment with the permeabilizing solution, all sperm became non-motile within 30 sec. Upon addition of ATP into the permeabilizing solution, instant flagellar beating was observed and prolonged up to 24 min. The maximal beating of the flagella was initiated in the presence of 1 mM ATP (Fig.4). To explore if Na^+/K^+-ATPase, dynein ATPase, creatine kinase and phosphodiesterase were involved in the motility of tilapia sperm, both flagellar beating and these enzyme activities were determined in the presence of various concentrations of each enzyme inhibitor. The sperm possessed Na^+/K^+-ATPase, dynein ATPase, creatine kinase and phosphodiesterase with specific activity of 20.8, 76.2, 794.5 and 0.02 nmol/min/mg protein, respectively. Ouabain, sodium vanadate, FDNB and 1-methyl-3-isobutylxanthine strongly inhibited the activity of Na^+/K^+-ATPase, dynein ATPase, creatine kinase and phosphodiesterase, respectively with a half maximal inhibition ($K_{0.5}$) of 1.6 mM, 25 μM, 1.8 μM and 0.20 mM, respectively (Table 2). In a similar manner, these inhibitors pronouncedly inhibited the flagellar beating with $K_{0.5}$ of 1.3 mM for ouabain, 25 μM for sodium vanadate and 1.6 μM for FDNB (Table 2 and Fig.5). In contrast, 1-methyl-3-isobutylxanthine, a potent inhibitor of phosphodiesterase, activated the flagellar beating with $K_{0.5}$ of 0.24 mM (Table 2 and Fig.5).

Table 1 Effects of artificial freshwater, filtered freshwater and distilled water on sperm motility.

	Motility
Artificial freshwater	73%
Filtered freshwater	43%
Distilled water	33%

The values represent the mean of 3 animals.

Table 2 Effects of inhibitors and activator on flagellar beating and enzyme activity of sperm.

	$K_{0.5}$	
	Flagellar beating	**Enzyme activity**
Inhibitors		
Sodium vanadate	25.0 μM	25.0 μM
Ouabain	1.3 mM	1.6 mM
1-Fluoro-2,4-dinitrobenzene	1.6 μM	1.8 μM
1-Methyl-3-isobutylxanthine	-	0.2 mM
Activator		
1-Methyl-3-isobutylxanthine	0.24 mM	-

The values represent the mean of 2 animals.

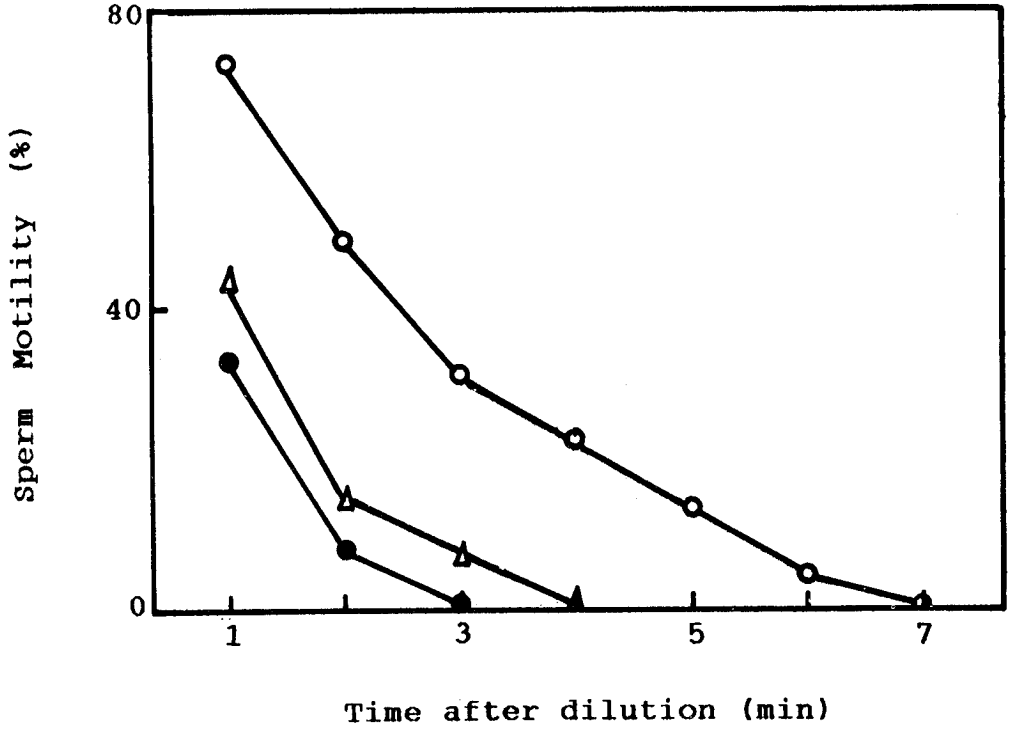

Fig.1 Sperm motility after dilution into artificial freshwater (o–o), filtered freshwater (Δ–Δ) and distilled water (●–●) as a function of time. The values represent the mean of 3 animals.

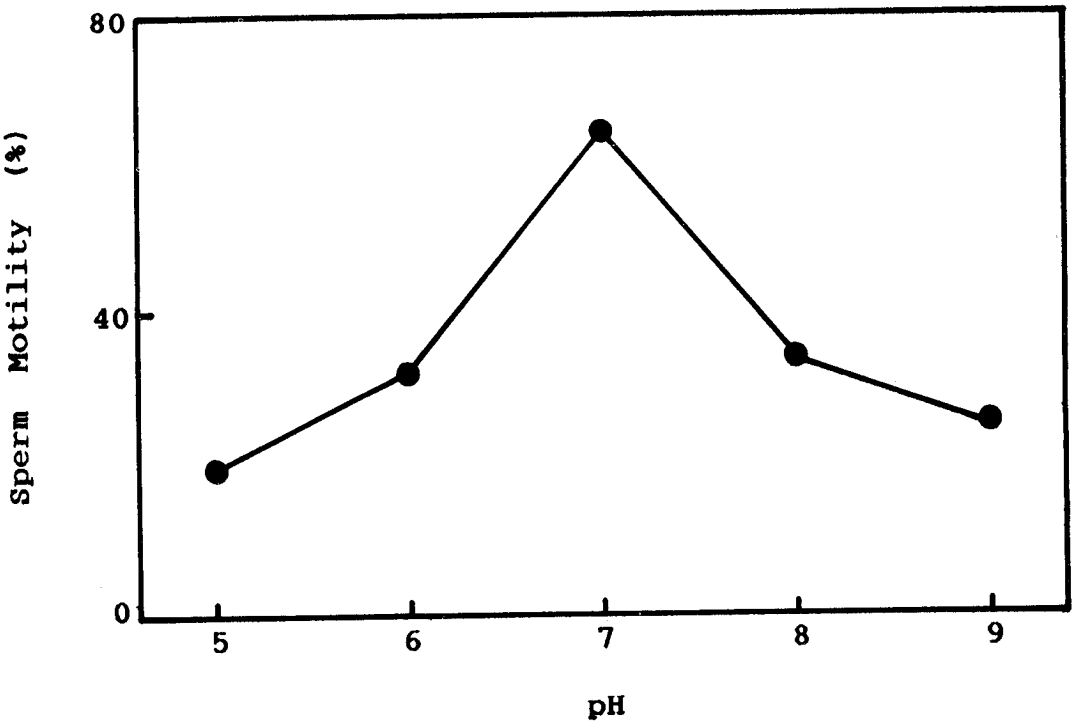

Fig.2 Effect of external pH on sperm motility. The values represent the mean of 3 animals.

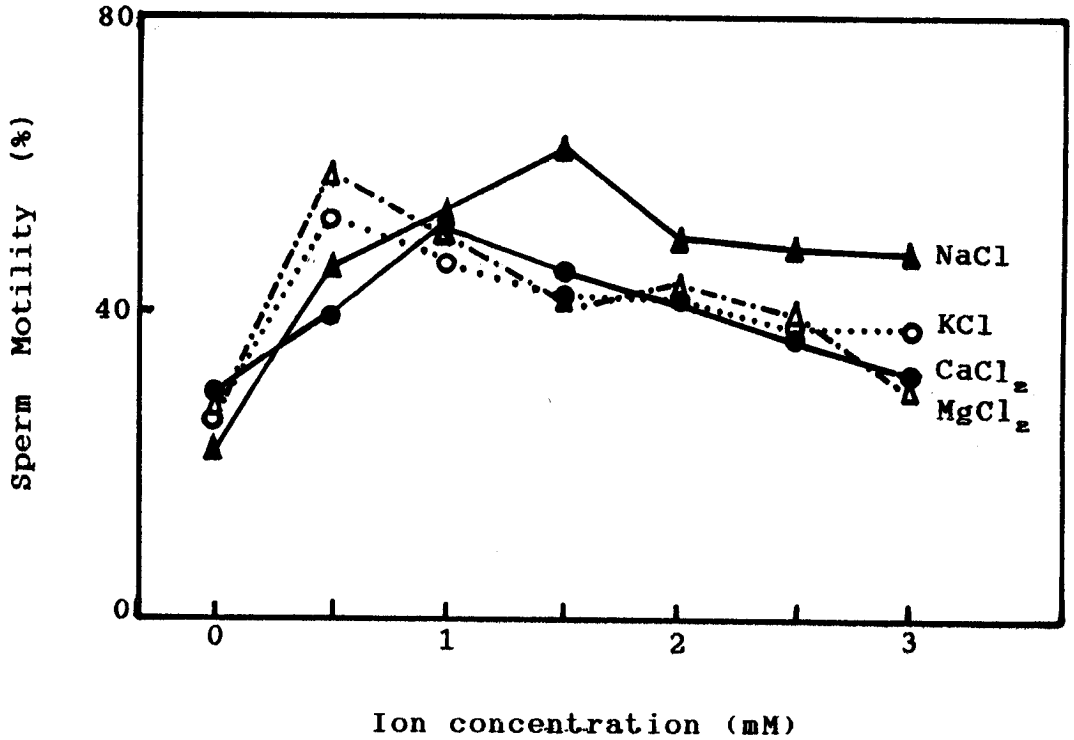

Fig.3 Effect of cations on sperm motility. The values represent the mean of 2 animals.

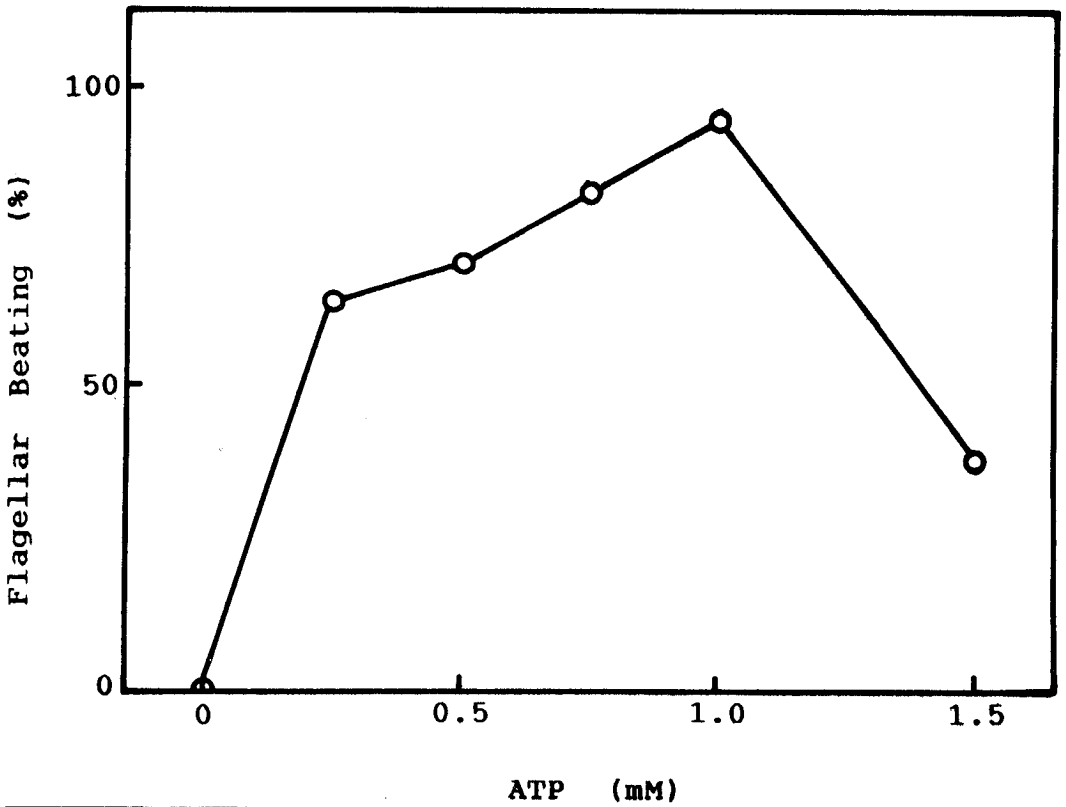

Fig.4 Effect of ATP concentrations on flagellar beating. The values represent the mean of 3 animals.

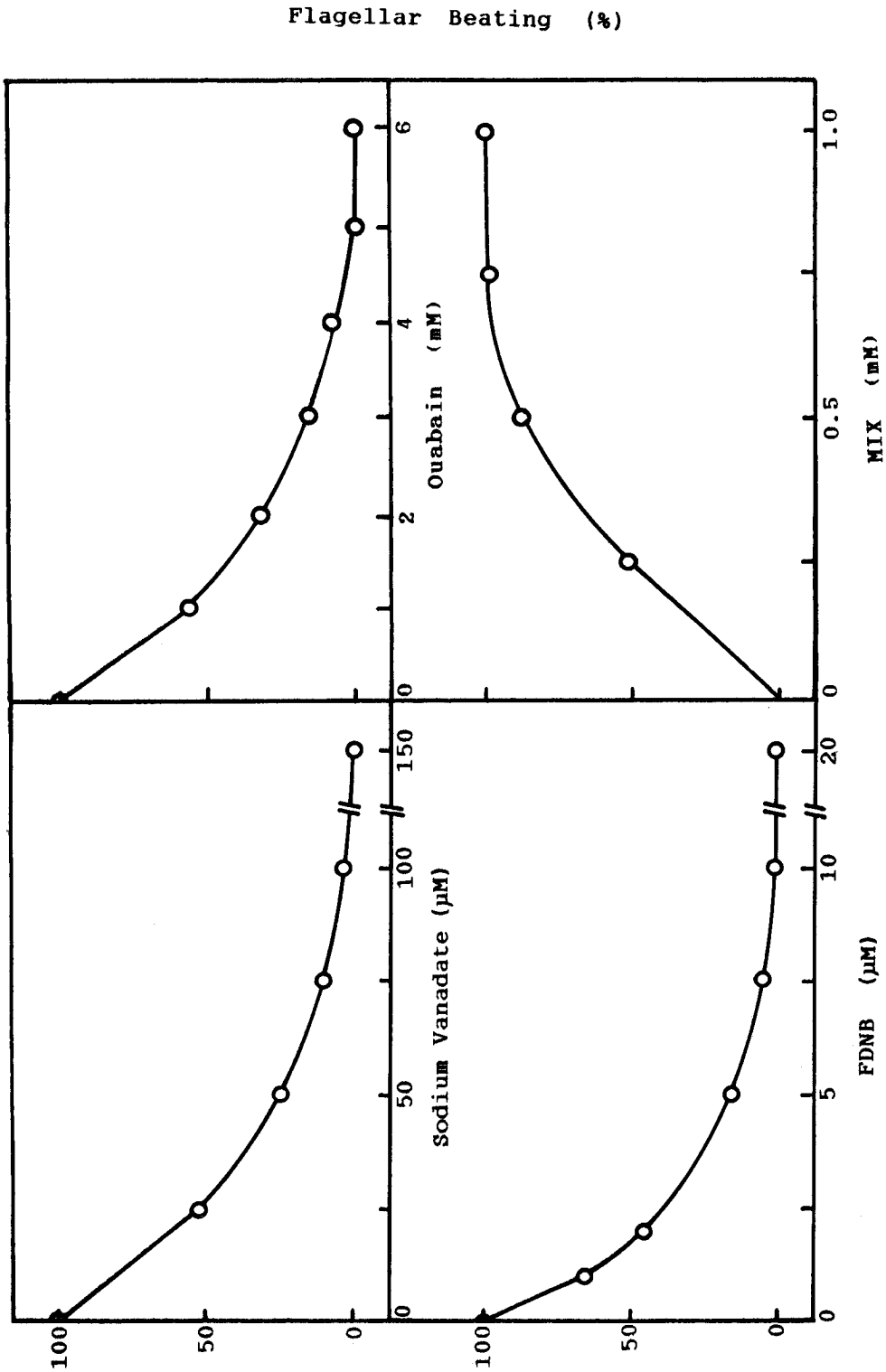

Fig.5 Effect of various levels of sodium vanadate, ouabain, 1-fluoro-2,4-dinitrobenzene (FDNB) and 1-methyl-3- isobutylxanthine (MIX) on flagellar beating. The values represent the mean of 2 animals.

DISCUSSION

In the gonads sperm are stored in a quiescent state.[26] Upon extrusion from the testes, all tilapia sperm were non-motile but became immediately motile upon dilution (1:200). Fish semen is vicous and not easily mixed with diluent[5] and a relatively high dilution (1:1000) was necessary to initiate simultaneous motility of 100 % sperm. In our investigation, uniform suspensions of testicular tilapia sperm were obtained with 1:200 dilution which may reflect a lower viscosity of testicular fluid than those of fish seminal fluid. Artificial freshwater has been shown to be suitable for the motility of rainbow trout[2] and in this study appeared better than the filtered freshwater or distilled water in activating the motility of the tilapia sperm (Table 1) with the reference that some ions may be needed for sperm activation. However high external concentration of Na^+ or K^+ or low concentration of Ca^{2+}, H^+ or Mg^{2+} cause a reduction in freshwater fish sperm motility (5, 32). All the ions tested individually [Na^+ (1.5 mM), K^+ (0.5 mM), Ca^{2+} (1.0 mM) or Mg^{2+} (0.5 mM)], at the same concentration as those composed in artificial freshwater, activated the highest motility of the tilapia sperm (Fig.3) and the same ions were shown to initiate motility of rainbow trout sperm.[2] However Na^+ is required to activate sea urchin sperm[30] and high concentration of K^+, present in seminal fluid, has been shown to inhibit the motility of spawning trout sperm[34] but not testicular sperm which appears to be less sensitive to inhibition by K^+.[5] In contrast, K^+ at moderate concentration can increase the motility of carp sperm[27] and there are several observations which indicate that external Ca^{2+} is also necessary for the initiation of motility. Kreimer *et al.*[22] report that Ca and calmodulin control sperm motility of sea urchin. Our investigation confirms the other reports that Na^+, K^+, Ca^{2+} and Mg^{2+} are all required for motility of tilapia sperm.

The optimal pH required for motility of tilapia sperm was pH 7.0 (Fig.2). Trout sperm motility was inhibited in medium of pH lower than 7.5 but Ca^{2+} and Mg^{2+} can overcome this inhibition.[4] Dilution of sea urchin sperm in seawater of low pH inhibited respiration and motility.[21,31] Low intracellular pH results in inhibition of both respiration and motility. Since the intracellular pH of sperm is affected by both extracellular pH and extracellular Na^+ concentration, part of the regulation of intracellular pH involved Na^+-dependent H^+ movement.[25,30] Upon dilution into artificial freshwater, pH 7.0, Na^+-dependent H^+ effux might lead to an increased intracellular pH which might act as an intracellular messenger to regulate the activation response in sperm as have been reported in sea urchin sperm,[10] rat sperm[38] and in tilapia sperm.[7]

The Na^+/K^+-ATPase activity of tilapia sperm is similar to the enzyme level in many somatic tissues and in *Strongylocentrotus purpuratus* sperm and with regard to its sensitivity to ouabain inhibition. The enzyme, and thus the Na^+ pump that generates a K^+-dependent sperm plasma membrane potential of -35 mV[33] is present uniformly throughout the sperm surface.[37] This plasma membrane potential is important for intracellular pH regulation.[23,24]

Inhibition of motility by treatment of *S. purpuratus* sperm with permeabilizing solution has also been reported by Gibbons and Rowe[18] but flagellar beating was maintained. Flagellar beating of tilapia sperm could be reactivated as a funtion of ATP concentration, with an optimum at 1 mM ATP. The sperm of most fishes spawning in freshwater rarely remain motile for longer than 2-3 min[20] whereas the flagellar movement of the permeabilized tilapia sperm in the presence of exogenous ATP was prolonged up to 24 min. This finding suggests that as for other species such as trout and carp,[5,9] halibut,[3] sea urchin[36] and rat[38] ATP is the energy source for motility of tilapia sperm.

Motility of sperm is sustained by the dynein ATPase of the flagellar axoneme[16] and by creatine kinase.[36] Both respiration and motility are impaired in the presence of either sodium vanadate or FDNB.[35,36] The inhibition of flagellar beating for tilapia sperm occurred with a half

maximal inhibition at concentrations similar to those shown to inhibit creatine kinase or dynein ATPase activity, indicating their involvement in the motility of the tilapia sperm.

A cAMP-dependent stimulation of sperm motility reportedly occurs for several species.[6,28] Both adenylated cyclase and cAMP phosphodiesterase are primarily located in sperm tails where the specific activity is about 4 to 7-fold higher than in isolated heads.[37] Exogenously added phosphodiesterase inhibitors, papaverine and caffeine, have marked effects on the motility of bovine sperm.[14] It is interesting that 1-methyl-3-isobutylxanthine activated the flagellar movement of the tilapia sperm with a $K_{0.5}$ of 0.24 mM whereas inhibited phosphodiesterase activity with a $K_{0.5}$ of 0.20 mM. This finding suggests that the phosphodiesterase enzyme negatively involves in sperm motility.

In conclusion, we evaluated here that motility of tilapia sperm was dependent on ion content and pH of diluant. ATP was found to maintain flagellar beating of permeabilized sperm. The four enzymes studied involved in motility of tilapia sperm.

ACKNOWLEDGEMENTS

The authors thank Prof. Montri Chulavatnatol and Dr. Alan F. Geater for correction to the manuscript. This work was supported by Faculty of Science and Faculty of Graduate Studies, Prince of Songkla University.

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