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Relationship between sperm ATP content and motility of carp spermatozoa

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SUMMARY

Carp spermatozoa are immotile in seminal plasma or in saline solution of high osmolality (>400 mosmol kg^{-1}). These 'quiescent' spermatozoa initiate a progressive forward motility when transferred in freshwater or in saline solution with low osmolality (<160 mosmol kg^{-1}). In this study we investigated 'in vitro' the relationship between sperm ATP content (measured by bioluminescence) and sperm motility (analysed by videomicroscopy). Sperm ATP content remained high in the immobilizing medium (200 mM KCl, Tris 30 mM, pH 8.0) where no flagellar movement occurs. Dilution of these spermatozoa in the activating medium (45 mM NaCl, 5 mM KCl, Tris 30 mM, pH 8.0) triggered forward motility which varied with temperature. At 20°C, sperm ATP content decreased rapidly during the progressive forward motility phase from 12 to 4 nmol/10^8 spermatozoa, concomitantly with decreases in velocity (130 to 10 μm s^{-1}) and the beat frequency (50 to 7 Hz). An inhibitor of mitochondrial respiration (KCN 10 mM) produced a drop in sperm ATP content irrespective of the incubation medium (activating or immobilizing). A second phase of sperm motility in the activating medium was induced following a previous transfer of spermatozoa into a medium of high osmolality for a few minutes prior to the second phase. Within 10 minutes, spermatozoa recover 90% of the initial ATP level as well as forward motility. These results suggest that motility of carp spermatozoa depends on sperm ATP synthesized by mitochondrial respiration mainly stored before activation. In low osmolality conditions, the mitochondrial oxidative phosphorylation is unable to compensate for the ATP hydrolysis required to sustain motility. The increase in osmolality surrounding spermatozoa probably blocks the dynein ATPases and allows an ATP 'regeneration' as a result of mitochondrial respiration.

Key words: spermatozoa motility, mitochondrion, bioluminescence, fish

INTRODUCTION

In most aquatic species with external fertilization, spermatozoa are immotile in the testis and become motile at release into the external medium. Most of the studies on regulation of motility and flagellar movement involved sea urchin spermatozoa. The latter are released into sea water where they become motile for at least one hour, the sperm flagellar beat triggered mostly by a rise in internal pH value. When intracellular pH is below 7.5, sperm cells remain immotile with low respiration rate, but in response to an internal alkalinization, they become motile, concomitantly with an increase in oxygen consumption (Christen et al., 1983; Shapiro et al., 1985). Under these conditions, there is an equilibrium between the ATP synthesis by mitochondria and its hydrolysis by dynein ATPases (Christen et al., 1982; Lee et al., 1983).

Despite structural similarities (mitochondrion located in the posterior part of the head and a '9+2' axonemal structure), the behavior of fish spermatozoa appears very different from that of sea urchin sperm. In most fish species, spermatozoa have a short period of progressive motility (from 30 seconds to several minutes) (Scott and Baynes, 1980) after release into the external medium. The intracellular mechanisms involved in immobilization or activation are not well known in fish sperm. Most currently available information on motility of fish spermatozoa originates from spermatozoa of two freshwater fish species: trout and carp. In trout, motility is inhibited in late spermatids and early spermatozoa by a low external calcium concentration in the testes (Billard and Cosson, 1992), and in mature spermatozoa by a high concentration of K^+ in the testes and sperm duct (Morisawa and Susuki, 1980; Baynes et al., 1981). Initiation of the progressive motility is associated with a rise in intracellular calcium concentration resulting from entry of Ca^{2+} (Tanimoto and Morisawa, 1988; Cosson et al., 1989; Boitano and Omoto, 1992). It appears that ionic transport across the membrane caused by a change in membrane potential can trigger the initiation of motility (Boitano and Omoto, 1988; Gatti et al., 1990). The period of motility is short (20-25 seconds) and flagellar beat frequency declines from 60 to 20 Hz just before the end of forward movement (Cosson et al., 1985). With decreases in temperature, sperm beat frequency is lower and the duration of motility is longer

(Billard and Cosson, 1988). During the propulsive motility phase, ATP exhaustion parallels the decrease of flagellar beat frequency (Christen et al., 1987; Robitaille et al., 1987; Billard and Cosson, 1990).

In carp testes and seminal plasma, inhibition of sperm motility is due to high osmolality (approximately 300 mosmol kg^{-1}) surrounding spermatozoa (Morisawa et al., 1983a; Redondo-Müller et al., 1991). Motility is initiated after a decrease in osmolality by dilution of spermatozoa in freshwater or in a saline solution (45 mM NaCl, 5 mM KCl, Tris 30 mM, pH 8) and lasts 45 seconds to 1.5 minutes (Redondo-Müller et al., 1991). A progressive decline in beat frequency is also observed (Cosson et al., 1985; Billard and Cosson, 1992). After hormonal stimulation, the volume of carp semen, the osmolality of seminal plasma and the capacity of sperm to move are very variable (Redondo-Müller et al., 1991). The potential ability for movement was preserved upon dilution of semen in a cold saline solution (NaCl or KCl) combined with high osmolality: 300-400 mosmol kg^{-1}. During storage in such a medium, called 'maturation medium', spermatozoa with initial 'poor' capacity to move gradually recover their ability to initiate movement. For preservation of freshly collected milt and 'in vitro maturation', spermatozoa need at least 50 mM KCl in medium with high osmolality. Although NaCl has properties similar to that of KCl, the recovery of ability for motility is faster with KCl. Activation of carp spermatozoa is not dependent on external pH in a broad range (6.0-10.0) (Redondo-Müller et al., 1991). In cases where carp spermatozoa show poor motile capacity, they always initiate progressive motility after demembranation and reactivation with MgATP, indicating that the axonemal machinery is functional, thus excluding intracellular proteolysis as a cause for the poor motility of intact spermatozoa. Such reactivation of demembranated carp spermatozoa requires no cAMP (Cosson and Gagnon, 1988).

In the present study changes in carp sperm movement characteristics (percentage of motility, velocity and flagellar beat frequencies) and sperm ATP content were investigated together with their response to environmental factors such as osmolality and temperature. In addition cyanide was used as a metabolism blocker, to investigate the capacity for mitochondrial oxidative phosphorylation of carp spermatozoa.

MATERIALS AND METHODS

Animals

Male carp (*Cyprinus carpio)* weighing between 0.9 to 1.5 kg were obtained from INRA (Jouy en Josas, France), and from two private fish farms: Mrs de Montalembert (Morvan, France) and de Courson (Champagne Ardennes, France). Carp were individually identified with colored tags and kept in 2 m^3 tanks under natural photoperiod and temperature. Water was renewed twice daily (flow rate: 3 liters/minute). Fish were fed with trout pellet every other day.

Sperm collection

Spermiation was induced every week by intraperitoneal injection of carp pituitary extract (Argent Chemical Laboratories), at a dose of 2 mg kg^{-1} body weight. Spermiation started 12-24 hours later, and semen was collected daily for the next three or four days. Fish were anaesthetized by immersion for few minutes in 2-phenoxyethanol at a dose of 0.5 ml l^{-1} of water. Contamination of milt with water, urine or faeces was carefully avoided. Semen was collected by abdominal pressure into separate collecting tubes of 5 ml and immediately placed on ice. The semen was not pooled. Experimentation started within the following 30 minutes of collection.

Sperm preparation

Because carp semen has a high sperm concentration (4 to 30×10^9 spermatozoa ml^{-1}) and high viscosity, direct uniform dispersal in the diluent is difficult. Therefore, observations were made after a two-step dilution. An aliquot of semen was first diluted 100-fold in a test tube containing a solution of 200 mM KCl, 30 mM Tris-HCl, pH 8.0, osmolality >400 mosmol kg^{-1} in which spermatozoa are maintained quiescent (IM, immobilizing medium) (Morisawa et al., 1983a). The second 20-fold dilution was made in an activating medium (AM) with low osmolality (<160 mosmol kg^{-1}) 5 mM KCl, 45 mM NaCl, 30 mM Tris-HCl, pH 8.0 (Saad and Billard, 1987) in a test tube for ATP measurement (see below) or directly on a glass slide (coated with 1% BSA), without coverslip, previously placed on the stage of the microscope for motility analysis.

In the experiments with cyanide, the final concentration in IM and AM was 10 mM KCN from a 0.5 M stock solution. For the regeneration, six minutes post-activation, 90 µl of KCl 1 M was added in AM to reach an osmolality around 300 mosmol kg^{-1}. The final concentration was 96 mM KCl in 'regenerating medium'.

Sperm motility analysis

An estimation of the percentage of motile spermatozoa was first made on the whole drop before any experiments in order to select 'good' sperm with a high percentage (>90%) of spermatozoa with progressive forward motility. The precision was in the range of ±5% confirmed by measurement with still-frame video.

The movements of carp spermatozoa were recorded at 25 frames per second using a camcorder (Canovision EX 1 Hi, Canon) fixed onto a dark-field microscope (Olympus BH-L obj. ×20) illuminated by a stroboscope light source (Chadwick Helmut Strobex). The focal plane was always positioned near the glass slide surface. Spermatozoa were visualized on a video monitor (Panasonic BT-M1420PY) with a ×750 final magnification on the screen. All video recordings were made at room temperature and recordings were analyzed frame by frame, the time interval between two frames was 40 milliseconds.

Velocity of spermatozoa was determined at various time points post-incubation in a medium supporting sperm activation. The successive positions of the sperm heads of ten different spermatozoa were drawn on transparent paper placed on the screen to allow tracking analysis of head trajectories. Spermatozoa were randomly chosen from an homogeneous population. Flagellar beat frequency was measured with the stroboscopic flash illumination. For technical reasons, precise velocity and beat frequency determinations could not be obtained at 2°C.

ATP determination

ATP was extracted from carp spermatozoa and measured by bioluminescence using a Biocounter M2010A Lumac/3M. For ATP measurements, the sperm suspension was kept in a test tube. The sperm extract samples were prepared as recommended by Fiorelli et al. (1982) and Orlando et al. (1982), and were measured using the protocol described for rat spermatozoa (Jeulin and Soufir, 1992). Briefly, an aliquot of sperm suspension was lysed by dilution into boiling buffer (25 mM HEPES, 10 mM magnesium acetate, 2 mM EDTA, 3 mM sodium azide, pH 7.75). The sperm extracts were kept frozen in plastic test tubes at –20°C. The procedure for ATP determination involved addition of a mixture of 100 µl of purified luciferin-luciferase (Kit from Perstorp S. A., Division Lumac, Bezons, France) freshly diluted in the above-mentioned Hepes buffer medium to 100 µl of sperm extract and measurement of luminescence. The ATP content of each sperm sample was calculated using the internal standard method in which 10 µl of known concentration of ATP

(Perstorp S. A., Division Lumac, Bezons, France) were added to the assay. Each extract was measured in duplicate. Sperm concentration was determined with a Thoma haemacytometer. Results were expressed as nmol ATP/10^8 spermatozoa.

Statistical analysis used an analysis of variance with a significance level of 5% confidence (Statgraphics, STSC Inc, Rockville, USA).

RESULTS

Effects of temperature

Sperm movements in the activating medium (AM) were very different at 20(±2)°C (room temperature) than at 2(±1)°C (activation in a test tube placed on ice). At room temperature, the percentage of motile spermatozoa showing a progressive forward motility remained high (95%) and was stable during the first 10 minutes, then decreased. The decline in the percentage of motile spermatozoa occured much later on ice, more than 15-20 minutes after activation.

Flagellar beat frequency gradually decreased from 50-60 Hz to 7-10 Hz for motile spermatozoa at 20°C (Fig. 1). This decrease was slower at 2°C compared to 20°C. Even if spermatozoa were immotile after the progressive forward motility, their flagella were still beating at low frequency (<3-4 Hz) as the total arrest of any movement after more than 30 minutes post-activation, at room temperature.

Video analysis of sperm head tracks (Fig. 2) showed a decline in speed during the period of progressive motility. At 20°C, the mean velocity of spermatozoa was 130±11 μm s^{-1} 10 seconds after dilution, but declined sharply and reached values of 16±8 μm s^{-1} 2 minutes post-activation and then remained practically stable (around 10 μm s^{-1}) for about the next 10 minutes where spermatozoa were still motile and progressive. The mean velocity for the first two minutes of displacement was 47 μm s^{-1}, and the mean total distance covered by a spermatozoon was 5.7 mm.

The ATP levels of immotile spermatozoa maintained at 2 and 20°C in IM were similar and remained constant for nearly an hour (Fig. 3A). In all cases, motility evaluation (dilution in AM) revealed an homogeneous population of motile spermatozoa (95%). The intermale variability in ATP content was low for both sperm populations kept at 20°C (10.7±0.2 nmol/10^8spermatozoa) and 2°C (11.8±0.9 nmol/10^8spermatozoa).

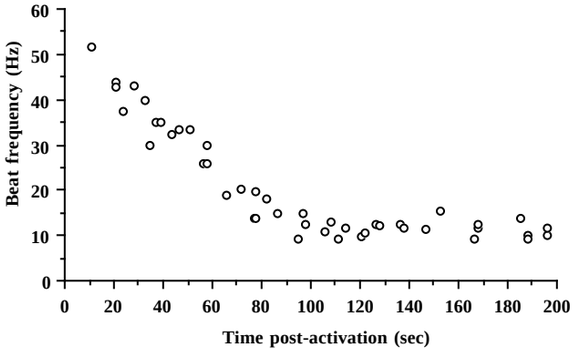

Fig. 1. Flagellar beat frequency of carp spermatozoa with progressive forward motility. Sperm were diluted 2000-fold in the activating medium (AM) at room temperature (20(±)2°C). Beat frequency was assessed as describe in Materials and Methods. Each point represents a determination from an individual spermatozoon and data from one sample.

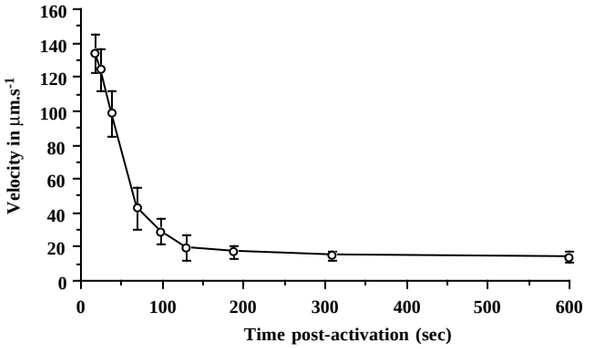

Fig. 2. Velocity measured in carp spermatozoa after activation in AM at 20(±)2°C. Mean±s.e.m. for five experiments performed and ten spermatozoa per sample observed. Velocity was obtained from the analysis of head trajectories; spermatozoa were followed during one second at various time points following the initiation. Films were analysed on a video display at 40 millisecond intervals between two frames.

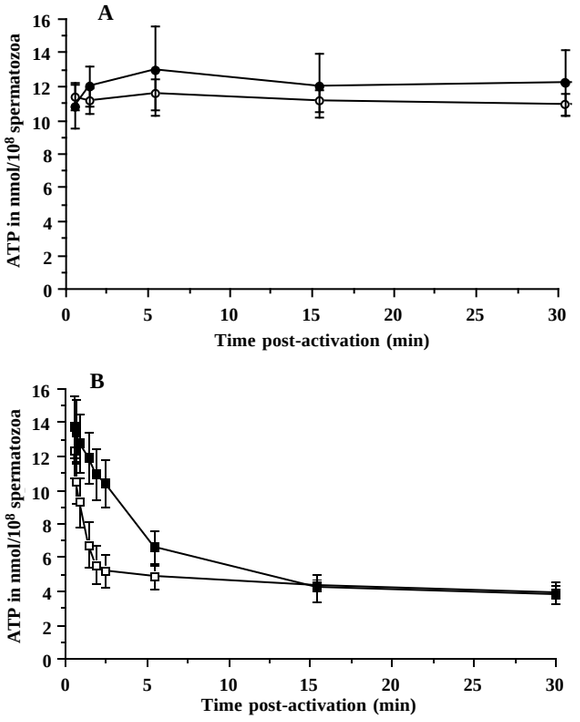

Fig. 3. Effect of temperature on sperm ATP content on immotile and motile spermatozoa was followed during 30 minutes. (A) Sperm were diluted 2000-fold in IM at 2°C (●) or at 20°C (○) (mean±s.e.m., *n*=3). (B) 2000-fold dilution in AM at 2°C (■) or 20°C (□) (mean±s.e.m., *n*=4).

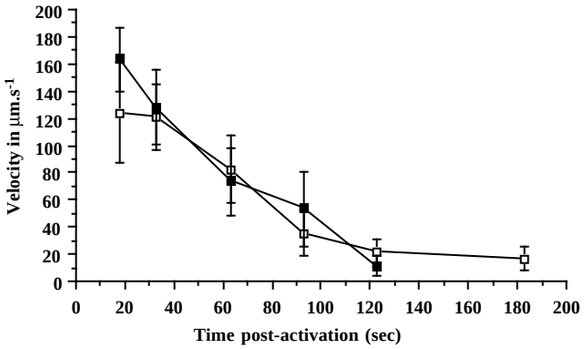

Fig. 4. Velocity of carp spermatozoa diluted 2000-fold in AM with (■) or without (□) 10 mM cyanide an inhibitor of mitochondrial respiration. Cyanide was mixed with AM just before addition of spermatozoa. Velocity was measured as already described in Fig. 2 (mean±s.e.m., *n*=3).

The ATP level during the motility phase (dilution in AM) showed a rapid decrease within two minutes followed by a plateau until 5 minutes at values of 4.4±0.7 nmol/10^8spermatozoa at 20°C (Fig. 3B). At ice temperature, the rate of decrease was slower. Cells had hydrolysed 60% of their ATP content after 2 minutes at 20°C; whereas within the same period, at ice temperature, only 30% of the sperm ATP content was hydrolyzed. Differences in sperm ATP levels at these two temperatures were significant until 5 minutes (P<0.05). All subsequent experiments were performed at 20°C which is the physiological temperature of fertilization in carp.

Effect of inhibition of mitochondial respiration

In the presence of 10 mM KCN in AM and without preincubation in IM there was no significant (P<0.05) change in motility parameters during the first seconds post-dilution. Some spermatozoa displayed an abnormal behaviour and showed erratic movements after one minute in AM containing 10 mM KCN whereas other sperm cells were affected much later. The total period of progressive motility was reduced in the presence of the inhibitor (Fig. 4) and when sperm heads were immotile, flagella had lower beat frequency and stopped more rapidly.

Immediately after dilution in IM, sperm ATP content was unaffected by cyanide: 10.8±0.5 nmol/10^8spermatozoa (without KCN) and 11.0±0.5 nmol/10^8spermatozoa (with 10 mM KCN) (Fig. 5A). The effect of cyanide on ATP depletion became significant after 10 and 15 minutes of exposure (P<0.05). In the presence of the inhibitor, sperm ATP content decreased slowly by 75% to 2.9±0.6 nmol/10^8spermatozoa after one hour. In control without cyanide sperm ATP content remained constant.

Sperm ATP contents of motile spermatozoa with or without 10 mM cyanide during the first phase of the activation were similar. However, in the presence of cyanide, ATP levels fell more rapidly (Fig. 5B). The action of the inhibitor was visible at 30 seconds to one minute post-dilution in AM (P<0.05). After 30 minutes, spermatozoa had exhausted 75% of their initial ATP reserve without cyanide, and 85% in the presence of cyanide; after one hour 80% and 95%, respectively.

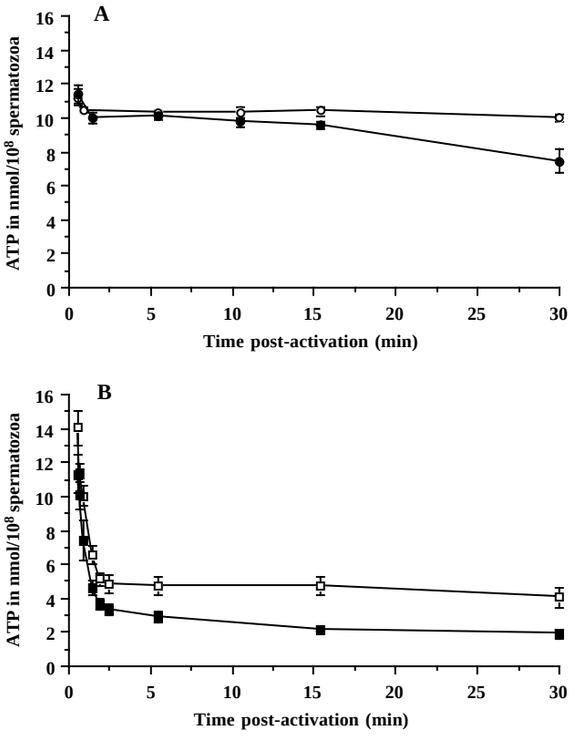

Fig. 5. Effect of cyanide on sperm ATP content in carp spermatozoa at 20(±)2°C. (A) Sperm were diluted 2000-fold in IM with (●) or without (○) cyanide 10 mM. (B) 2000-fold dilution in AM with (■) or without (□) cyanide 10 mM (mean±s.e.m., *n*=4).

Remobilization

After six minutes of motility activation, flagellar activity was stopped by addition of 1 M KCl raising the osmolality to 300 mosmol kg^{-1} (regenerating medium). At various time period after immobilization, a second motility phase was initiated by dilution in AM. Velocity of spermatozoa increased gradually and regained the values attained during the first motility phase (116±6 μm s^{-1} after 10 minutes and 122±11 μm s^{-1} after 15 minutes of exposure to the 'regenerating medium').

Immediately after addition of KCl to the AM suspension, sperm ATP content increased (Fig. 6). The maximal value was obtained 5-10 minutes later and remained at that level for 15 minutes before decreasing slightly. However, sperm ATP content measured after 10 minutes of exposure to the 'regenerating medium' was lower (8.1±0.6 nmol/10^8spermatozoa) than that of spermatozoa prior to the first initiation of motility (9.1±0.5 nmol/10^8spermatozoa), thus spermatozoa had recovered around 90% of their initial sperm ATP content under these conditions.

DISCUSSION

In the present work, carp sperm movement was studied in relation to the energy store under various conditions. Flagellar beat frequency and velocity were analysed using methods (video analysis, stroboscopy) similar to those used for sperm

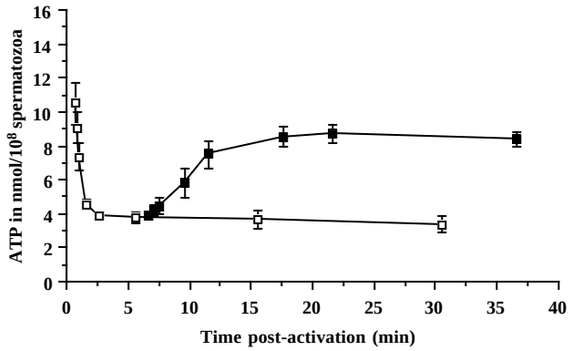

Fig. 6. Effect of a rise in osmolality on ATP content of motile spermatozoa. Spermatozoa were diluted 2000-fold in AM (□) and six minutes later they were placed in high osmolality conditions (■) (300 mosmol kg^{-1}) by addition in AM of 90 µl of KCl 1 M (mean±s.e.m., *n*=4).

flagellar movement of sea urchin (Hiramoto and Baba, 1978), carp and trout (Cosson et al., 1985). Sperm ATP content was measured by a simple and precise bioluminescence method using the internal standard protocol, already applied successfully to rat spermatozoa (Jeulin and Soufir, 1992).

Motility of carp spermatozoa differs from that of the classical model, sea urchin sperm (Gibbons, 1981; Shapiro et al., 1985): the latter remain motile for hours after spawning in contrast to carp spermatozoa which show a short period of forward movement. In addition, rapid changes in motility parameters similar to those of trout (Cosson et al., 1989), halibut (Billard et al., 1993) and turbot (Suquet et al., 1992; R. Billard, unpublished data) were also observed. The kinetics of motility in AM depends on temperature. At 20°C, which is the temperature during spawning, carp spermatozoa have a shorter period of progressive motility. Parameters such as velocity and beat frequency decrease concomitantly and the flagellar waves become more and more flattened toward the tip of the flagellum. Wave generation becomes localized to the proximal part with the distal part remaining either rigid or reacting to the thrust of incoming waves. This confirms the preliminary data obtained by Billard and Cosson (1989). Progressive motility requires a beat frequency threshold of 3-4 Hz below which no net sperm progression occurs. At 2°C, the mechanochemical coupling was reduced: the duration of motility increased, but velocity and flagellar beat frequency were reduced.

Immediately after activation, velocity and beat frequency are similar in trout and carp spermatozoa but decrease more rapidly in the trout (Billard and Cosson, 1988; Cosson et al., 1989), this is different from halibut or turbot sperm which show an abrupt drop in beat frequency at 40 seconds (halibut) or at 60 seconds (turbot) (Billard et al., 1993; R. Billard, unpublished data). Finally, the total calculated distance covered by carp spermatozoa is higher (5-6 mm) than that of trout spermatozoa (2.5-3 mm). The efficiency of fertilization is therefore probably better in carp than in trout considering this longer distance of displacement and the smaller size of the oocyte (1-1.5 mm in carp, 4-5 mm in trout); the probability of spermatozoa meeting with the micropyle is higher.

Cyanide (10 mM), which blocks the energy production through mitochondrial respiration, decreased the total duration of carp spermatozoa displacement in AM and when sperm heads were immotile, flagellar beat frequency was lower or zero. At the end of a first motility phase, spermatozoa were transferred to the 'regenerating medium' (300 mosmol kg^{-1}) for a few minutes, then spermatozoa can have a second motility phase following their transfer into hypoosmotic conditions. In the case of carp spermatozoa, a modification in the activating medium is necessary to interupt the flagellar beat activation. In contrast, trout spermatozoa stopped abruptly 20-30 seconds after activation without change of medium, and can initiate a second motility phase 15 minutes later, following the addition of millimolar concentrations of calcium (Christen et al., 1987).

In the present study, carp spermatozoa had a nearly constant and high ATP content for at least one hour post-dilution in IM in which they are immotile irrespective of the temperature. When 10 mM cyanide (KCN) is added to immotile spermatozoa, their ATP content progressively decreases. In trout, a similar but faster decrease in ATP content was observed with cyanide (Christen et al., 1987). These results confirm the requirement of respiration for the maintenance of high ATP levels in both carp and trout spermatozoa. Thus constant ATP levels of spermatozoa in IM result from a dynamic balance between mitochondrial synthesis and ATP metabolizing enzymes (ATPases). During the first two minutes post-dilution in AM at 20°C, sperm ATP content decreases rapidly, and the average rate of ATP disappearance is high, at about 6 nmoles/10^8spermatozoa during the first minute and remains at a plateau between two to five minutes. The decrease in sperm ATP content is principally due to its hydrolysis by dynein ATPases coupled to movement. We have no explanation for the differences in sperm ATP levels between IM and AM at the begining of the dilution in these suspension media. In trout a high ATP utilization is also observed during the first minute but the endogenous store then increases immediately and spontaneously (Christen et al., 1987). The period of ATP decrease and that to plateau are much longer at 2°C. With cyanide, at 20°C, the decrease in ATP content is much faster. The rate of ATP synthesis under high osmolality ('regenerating medium') is around 0.7 nmol/10^8spermatozoa/minute. The ATP content recovers to initial values (pre-motility values) within ten minutes. In trout spermatozoa the spontaneous recovery of ATP required 15 minutes (Christen et al., 1987).

Motility of spermatozoa depends on the dynein ATPases which hydrolyse ATP to produce a flagellar beat (Gibbons, 1968). Even through carp spermatozoa are immotile, they have sufficient stores of ATP to sustain motility but they remain immotile. Some ATP metabolic enzymes are definitely active as evidence by the ATP decrease in IM in the presence of cyanide. These metabolic enzymes may include the ATPases which control ionic exchanges across the membrane and possibly also the dynein ATPases not coupled to movement. During activation, the change in motility parameters seems to be directly related to ATP content. All the measured parameters in this study are at their maximum value at the onset of motility. However, they rapidly decrease in the next several seconds to reach a plateau within minutes. ATP levels remain around 30% of the initial store at the end of the forward motility period suggesting a state of dynamic balance between ATP synthesis by mitochondria and its hydrolysis, mostly dynein ATPases. Mitochondrial activity in carp spermatozoa

is not sufficient to sustain a high endogenous store of ATP and to compensate the ATP utilization by ATPases coupled to movement. On the contrary, sea urchin spermatozoa have a high mitochondrial activity, allowing a level of ATP sufficiently elevated to sustain normal motility for periods of time more than an hour (Christen et al., 1983) similarly to mammalian spermatozoa (Jeulin and Soufir, 1992).

At the end of the progressive forward motility period, part of the ATP may be inadequately distributed to the entire length of the flagellum. Before the end of progressive motion, flagellar waves were present only in the proximal part near the mid-piece region which could reflect a progressive decrease of ATP shuttle molecules like phosphocreatine as shown in trout (Robitaille et al., 1987) and sea urchin (Tombes et al., 1987): ATP is not carried with sufficient efficiency to the distal part of the flagellum thus preventing wave propagation to the tip of the flagellum. In mammalian spermatozoa, ATP shuttle molecules were not observed probably as a consequence of the closer vicinity of mitochondria and flagellum (Jeulin and Soufir, 1992). It should be noted that in carp as in many other teleost fish, the flagellum is separated from the mitochondrion by the cytoplasmic canal in such a way that mitochondrial ATP is not directly in contact with the axoneme (Billard, 1969) and a shuttle should transport ATP close to dynein ATPases.

The decrease of flagellar motility after the displacement phase could result from cellular damage, but this hypothesis is not supported by the fact that a second motility phase can be initiated (see below). It is worth noting that at the end of the progressive motility phase, the addition of MgATP in AM did not extend nor reactivate movement as seen in spermatozoa of halibut partially damaged after cryopreservation (Billard et al., 1993): the plasma membrane remains impermeable to exogenous ATP and does not deteriorate as a result of osmotic shock occuring during initiation. Another additional explanation could involve an ADP accumulation (decrease of the ATP/ADP ratio) due to ATP hydrolysis by ATPases. ADP is a competitive inhibitor of ATP for the dynein ATPase and a high ADP concentration could decrease flagellar movement (Omoto, 1991, for review). In vitro, during reactivation of demembranated flagella with 0.3 mM MgATP and 1 mM ADP, carp spermatozoa showed erratic and limited flagellar movement (data not shown).

The internal acidification resulting from ATP hydrolysis could also lower spermatozoa velocity by decreasing the activity of dynein ATPases as a result of non optimum intracellular pH: in trout sperm, motile spermatozoa have increased mitochondrial activity allowing internal alkalinization leading to optimal pH for dynein ATPase activity but there is no relationship between intracellular pH and initiation of movement (Gatti et al., 1990; Boitano and Omoto, 1991). However, a modification in the pH gradient affects membrane potential and could change the Na^+ and K^+ permeability (Gatti and Christen, 1985; Boitano and Omoto, 1991). In carp spermatozoa, there is no information about the role of intracellular pH during activation, and the possibility of an inhibitory effect of the internal acidification can not be excluded.

When KCl was added in AM at any time after activation in such a way that osmolality reaches 300 mosmol kg^{-1}, carp flagellar beat stopped abruptly. In trout this abrupt stop occurs spontaneously at the end of the motility phase in the absence of Ca^{2+} and was interpreted as an axonemal and/or dynein ATPase switch off (Christen et al., 1987). In carp, transfer of actively motile spermatozoa in a high osmolality medium seems to block the axonemal machinery, allowing the regeneration of ATP. It should be the same in IM with high sperm ATP content and no movement. During regeneration, sperm ATP content increases rapidly leading us to suspect a respiratory activity higher than that of trout spermatozoa (Christen et al., 1987). In the case of regeneration, mitochondrial activity could be higher than during the first motility and/or the arrest of dynein ATPases could allow a rapid increase in sperm ATP content. The activation of motility in carp spermatozoa is a reversible phenomenon, switched on or off simply by external osmolality change, but after at least a partial ATP level regeneration.

In contrast to trout spermatozoa (Christen et al., 1987), carp spermatozoa do not require extracellular calcium for the second motility phase and after demembranation, the addition of Ca^{2+} to the reactivating medium is not necessary to initiate motility (M. P. Cosson, unpublished data). As in sea urchin spermatozoa (Brokaw et al., 1974), Ca^{2+} modulates the trajectory of carp spermatozoa through asymmetric beating but a role of intracellular calcium in the initiation of motility is not shown as it has been recently postulated for the marine teleost fish, puffer and flounder (Oda and Morisawa, 1993).

During motility, dynein ATPases hydrolyze the bulk of the ATP store: 70% of the total in bull spermatozoa (Rikmenspoel, 1965; Halangk et al., 1985). ATP could be also a source of cAMP as described in trout spermatozoa (Morisawa et al., 1983b). However, in carp spermatozoa cAMP does not play a major role in motility: demembranated carp spermatozoa can be ATP reactivated without cAMP addition in the medium in contrast to trout spermatozoa (Cosson and Gagnon, 1988). Membrane ATPases could also interfere to adjust the ionic or osmotic balance in response to the change from high to low osmolality. Moreover, spermatozoa can not sustain a high osmotic shock after a first motility phase (300 mosmol kg^{-1} instead of 400 mosmol kg^{-1} before motility). The change in osmolality may act on the ionic balance between intra- and extracellular medium as suggested by Redondo-Müller et al. (1991). In sea urchin, sperm motility is triggered by ionic movement accross the membrane (Darszon et al., 1987, for review). In trout, ionic exchanges across the plasma membrane have also be reported to be involved in activation (Gatti et al., 1990). Several authors propose that the ionic flux changes the membrane potential which triggers motility (Lee, 1984; Boitano and Omoto, 1991). The mechanism by which an osmotic shock could trigger motility of carp spermatozoa remains unknown. However, recent studies show that the hypo-osmotic shock induces a modification of membrane permeability and structural changes with a possible reorganisation of the lipid bilayer in this plasma membrane after transfer of spermatozoa from the semen environment to fresh water (Màriàn et al., 1993).

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