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Phosphatidylinositol 3-kinase inhibition enhances human sperm motility

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BACKGROUND: The number of spermatozoa with forward motility after capacitation procedures represents the limiting factor for application of IVF versus intracytoplasmatic sperm injection (ICSI) procedure in cases of oligoasthenozoospermia. The possibility of increasing this number may thus be of help to the patient. A complex array of signalling pathways is involved in the regulation of sperm motility and recent data pointed out an important role for kinase/phosphatase-regulated phosphorylation of proteins. Here, we investigated the role of phosphatidylinositol 3-kinase (PI3K), a lipid and protein kinase involved in the regulation of several biological aspects of somatic cells, on human sperm motility by using the specific PI3K inhibitor LY294002. METHODS AND RESULTS: We demonstrated that in-vitro incubation of swim-up selected or unselected human spermatozoa with LY294002 determined an increase of percentage forward motility in all the treated samples. The effect was dose-dependent with an EC_{50} of 1.09 ± 0.54 μmol/l. LY294002 also increased sperm movement characteristics and hyperactivation as evaluated by computer-assisted motion analyser. The compound was also able to overcome the detrimental effect of hydrogen peroxide and lithium chloride on sperm motility. CONCLUSIONS: Our results suggest a negative role for PI3K in the development and maintenance of sperm motility and suggest a possible use of PI3K inhibitors to enhance motility in cases of asthenozoospermia.

Key words: human/LY294002/motility/phosphatidylinositol 3-kinase inhibitor/phosphatidylinositol 3-kinase/spermatozoa

Introduction

The number of motile spermatozoa obtained after selection techniques is the limiting factor for application of IVF versus intracytoplasmatic sperm injection (ICSI) procedure in cases of moderate to severe oligoasthenozoospermia (Hamberger *et al*., 1998). However, the absolute safety of the ICSI procedure for the health of the conceptus is still a matter of debate (Edwards, 1999; Luetjens *et al*., 1999). In addition, ICSI is far more expensive than IVF. In this light, the possibility of increasing sperm motility in cases of asthenozoospermia might increase the chance of IVF application. Several pharmacological agents have been demonstrated to improve sperm motility (Lanzafame *et al*., 1994; Minhas and Ripps, 1996), including pentoxifylline (Tesarik *et al*., 1992) and platelet-activating factor (Krausz *et al*., 1994). However, the possibility of using such agents to increase the recovery of motile spermatozoa after selection is limited by the presence of non-responding subjects (Tesarik *et al*., 1992; Krausz *et al*., 1994), and in the case of pentoxifylline by the demonstration of toxic effects of the molecule (Centola *et al*., 1995). Knowledge of signal transduction mechanisms regulating sperm motility is of funda-

mental importance in developing new clinical tools to increase the fertilizing ability of semen samples. The role of cyclic AMP/protein kinase A (cAMP/PKA)-dependent pathway in sperm motility is well known, as also demonstrated by the increase of motility observed after inhibition of cAMP catabolic enzymes (Tesarik *et al*., 1992; Nassar *et al*., 1999). In addition, recent evidence pointed out a key role for protein kinases and phosphatases in the development of sperm motility (Tash and Bracho, 1994; Vijayaraghavan *et al*., 1997, 2000). However, the nature of most of the phosphorylated proteins as well as of the kinases responsible for such effects remains to be determined. Recently, the presence of phosphatidylinositol 3-kinase (PI3K) has been demonstrated in mouse spermatozoa (Feng *et al*., 1998). In addition, by using the PI3K inhibitor wortmannin, the possible involvement of PI3K in the induction of acrosome reaction by some known inducers of this event has been suggested (Fisher *et al*., 1998). PI3K catalyses phosphorylation in position 3 of the inositol ring of phosphatidylinositol, priming a series of signalling cascades involved in the regulation of a number of functions including mitogenesis, differentiation, motility and metabolic control (Wymann and Pirola, 1998; Anderson *et al*., 1999). Moreover, in somatic

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cells, PI3K can also phosphorylate some protein substrates (Wymann and Pirola, 1998; Anderson *et al*., 1999). We investigated here the role of PI3K in regulating the motility of human spermatozoa by using two unrelated PI3K inhibitors, LY294002 and wortmannin.

Materials and methods

Chemicals
Human serum albumin (HSA)-free human tubal fluid (HTF), HSA and TES buffer and Tris (TEST) yolk buffer were from Irvine (Santa Ana, CA, USA). Percoll was from Amersham Pharmacia Biotech Italia (Cologno Monzese, Italy). LY294002 and wortmannin were from Calbiochem (La Jolla, CA, USA). All the other chemicals were from Sigma (St Louis, MO, USA).

Preparation of spermatozoa
Human semen was collected, according to the WHO recommended procedure (WHO, 1999) by masturbation from 116 men undergoing semen analysis for couple infertility in our laboratory. Samples with leukocytes and/or immature germ cell concentration $>1\times10^6$/ml were not included in the study. Semen samples were processed by swim-up technique as previously described (Krausz *et al*., 1996). Briefly, 1 ml aliquots of semen were gently overlain with 1 ml of 1% HSA-HTF medium and incubated at 37°C, 5% CO_2. After 1 h, 800 µl of the upper medium phase were collected and checked for sperm count and motility. In 32 patients spermatozoa were selected by discontinuous density gradient centrifugation (WHO, 1999). Briefly, semen samples were layered on top of 95, 75 and 50% Percoll gradient and centrifuged at 500 *g* for 30 min. Only spermatozoa migrating in the lower phase were collected. After preparation, spermatozoa were incubated for 2 h with the PI3K antagonists at the indicated concentrations.

Cryopreservation and thawing of ejaculated spermatozoa
Ejaculated spermatozoa were frozen in liquid nitrogen in a Taylor–Wharton 34HC tank by manual controlled freezing procedure (Taylor–Wharton, AL, USA). Ejaculated spermatozoa were diluted 1:1 (v/v) by drop wise addition of TEST yolk buffer. After equilibration at room temperature for 5–10 min, the spermatozoa were loaded in 250 µmol/l straws. Straws were manually frozen by 8 min exposure to liquid nitrogen vapours and a final plunge into liquid nitrogen according to Taylor–Wharton procedure.

Thawing was carried out by leaving the straws at 37°C for 15 min. Motility and viability were assessed immediately after thawing.

Evaluation of sperm motility and viability
Motility was evaluated by phase contrast light microscopy according to WHO manual recommendations (WHO, 1999) at 37°C. At least 200 spermatozoa in five different microscopic fields were evaluated for each sample. In 12 samples sperm motility was evaluated by computer assisted semen analysis (CASA; Hamilton Thorn Research, Beverly, MA, USA) (Gandini *et al*., 1997). Criteria adopted to define hyperactivated motility were the following: linearity (LIN) ≤6.5; curvilinear velocity (VCL) ≥100 um/s; amplitude of lateral head displacement (ALH) ≥7.5 (Burkman, 1991). The settings used during the CASA procedure were: analysis duration of 1 s (30 frames); minimum contrast: 80; minimum size: 3; low size gate: 0.7; high size gate: 2.6; low intensity gate: 0.34; light intensity gate: 1.40. All measurements were performed at 37°C.

Sperm viability was evaluated under phase contrast light microscope by hyposmotic swelling test according to WHO (WHO, 1999).

Statistical analysis
Data are expressed as mean ± SEM. Statistical significance has been evaluated by Student's *t*-test for paired data. EC_{50} for the effect LY290042 on sperm motility has been evaluated by simultaneous analysis dose-response curves by the computer program ALLFIT (De Lean *et al*., 1978).

Results

The treatment of swim-up selected spermatozoa with LY294002 (10 µmol/l, 2 h) resulted in an increase of forward motility in sperm samples from all the subjects examined (Figure 1). Note that in many subjects, sperm motility 2 h after swim-up procedure lowered below 60%, and the treatment with LY294002 restored motility over 60% in most of these samples. On average, the percentage increase of forward motility after the treatment was of 38.7 ± 2.0. Similar results were obtained by using the structurally unrelated PI3K inhibitor wortmannin (not shown). However, since wortmannin has been shown also to inhibit other enzymatic activities (Cross *et al*., 1995; Ferby *et al*., 1996) including extracellular signal regulated kinases (Ferby *et al*., 1996), whose presence has been demonstrated in human spermatozoa (Luconi *et al*., 1998), subsequent experiments have been performed only with LY294002. The effect of LY294002 was dose-dependent (Figure 2) with an EC_{50} of 1.09 ± 0.54 µmol/l for rapid and of 0.83 ± 0.6 for rapid + slow motility. These values were determined by the simultaneous computer analysis of nine different experiments with the computer program ALLFIT (De Lean *et al*., 1978) and fall in the range of inhibitory concentration 50 (IC_{50}) of LY294002 on PI3K activity described in the literature (Vlahos *et al*., 1994). No effects on sperm viability were observed in any of the samples tested even after 48 h of incubation with LY294002 (not shown). When the treated sperm samples (Figure 1) were divided into normal and pathological according to basal semen parameter characteristics (WHO, 1999), and only oligo-asthenozoospermic subjects (*n* = 50) considered, the effect of LY294002 on forward motility was more evident (Figure 3) and the percentage increase of forward motility after the treatment was 67.6 ± 5.0. The stimulatory effect of LY294002 was also present when spermatozoa were selected with Percoll instead of swim-up technique (percentage sperm forward motility after 2 h of treatment: 47.6 ± 3.5 in control, 67.4 ± 3.1 in LY294002-treated, *n* = 32, *P* < 0.001).

To determine whether the increase of percentage motile spermatozoa observed with the two inhibitors was associated with changes in sperm movement characteristics and hyper-activated motility, these parameters were determined in 12 samples by CASA after 2 h treatment with LY294002. As shown in Figure 4, a significant effect of LY294002 was observed on VCL, average path velocity (VAP), straight-line velocity (VSL) and on percentage of hyperactivated sperm fraction (HA), whereas no significant effects were detected on other parameters such as linearity, lateral head displacement and beat cross frequency (not shown).

We next investigated the effect of LY294002 on sperm forward motility in seminal plasma of oligoasthenozoospermic
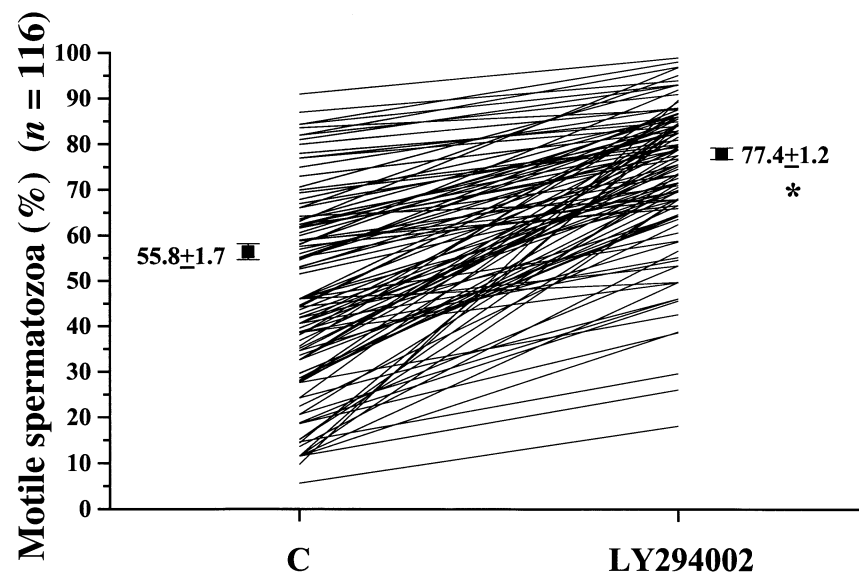
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Figure 1. Effect of 2 h incubation with LY294002 (10 µmol/l) on forward motility in swim-up selected human spermatozoa from 116 subjects. Mean ± SEM sperm forward motility before and after the treatment is also reported. *P < 0.001 versus control (C), *t*-test for paired data.
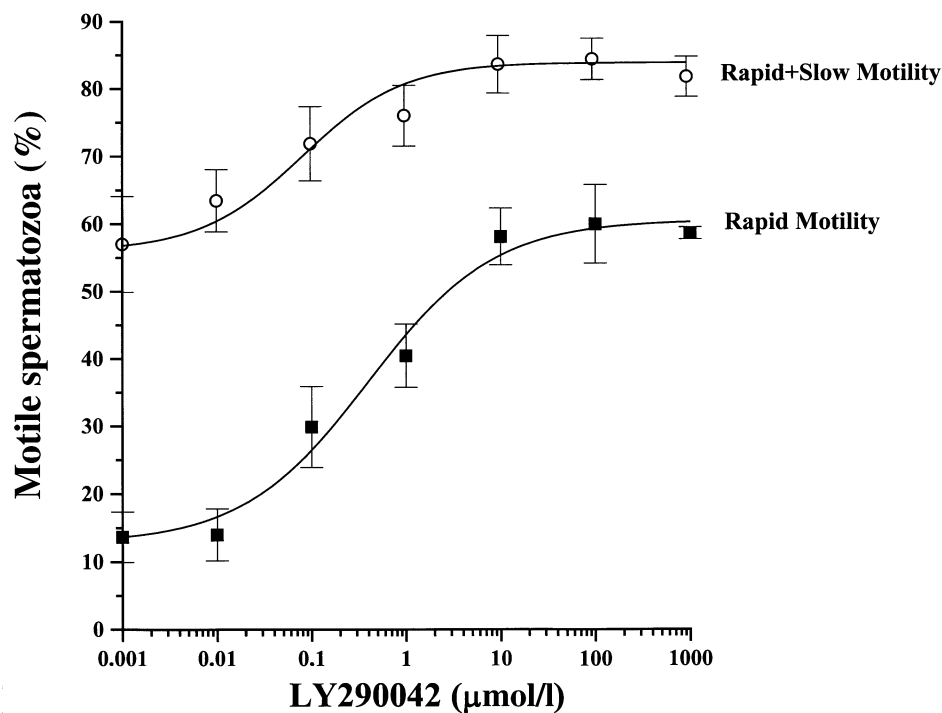

Figure 2. Dose-response curves of the effect of LY294002 on rapid (grade a) and rapid + slow (grade a + b) motilities (WHO, 1999) on swim-up selected human spermatozoa. Data represent mean ± SEM from nine different experiments analysed simultaneously with the computer program ALLFIT. EC_{50} were: 1.09 ± 0.54 µmol/l and 0.83 ± 0.60 µmol/l respectively for rapid and rapid + slow forward motilities.

subjects. As shown in Table I, at 10 µmol/l concentration, LY294002 significantly increased sperm forward motility when added directly to seminal plasma. The percentage increase of forward motility (39.0 ± 1.8) was similar to that observed in swim-up samples (Figure 1). The addition of LY294002 to seminal plasma slightly but significantly increased sperm recovery after swim-up in these subjects (Table I). Moreover, motility evaluated 2 h after swim-up was higher in spermatozoa selected from LY294002-containing seminal fluid than from

control (Table I), indicating that the effect of the inhibitor is maintained also after removal from the medium.

To investigate whether LY294002 could have a protective role toward loss of sperm motility *in vitro*, spermatozoa were treated with hydrogen peroxide (H_2O_2) and lithium chloride (LiCl), two agents known to affect motility strongly *in vitro* (Shen *et al.*, 1992; Griveau and Le Lannou, 1997). As expected, both agents significantly decreased sperm forward motility after 2 h treatment (Figure 5A,B) without affecting viability
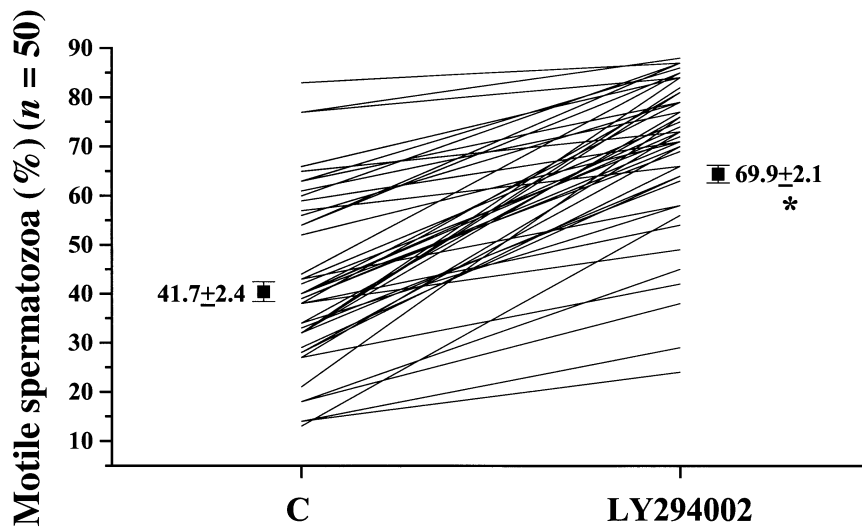
Figure 3. Effect of 2 h incubation with LY294002 (10 μmol/l) on motility in swim-up selected human spermatozoa from 50 oligoasthenozoospermic subjects. Mean ± SEM forward motility before and after the treatment is also reported. *$P < 0.001$ versus control (C), *t*-test for paired data.
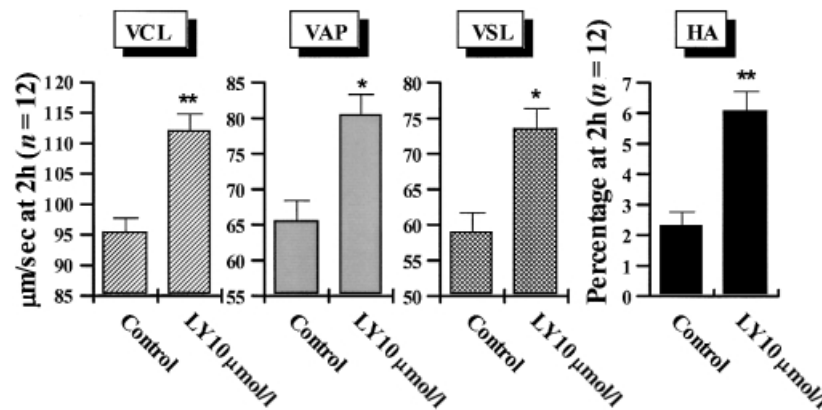

Figure 4. Effect of 2 h incubation with LY294002 (10 μmol/l) on sperm curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL) and percentage of hyperactivated (HA) spermatozoa in swim-up selected spermatozoa. $n = 12$ experiments. *$P < 0.005$, **$P < 0.001$, versus control, *t*-test for paired data.

Table I. Effect of LY294002 addition to seminal fluid on sperm motility and on number of spermatozoa recovered after swim-up procedure in 13 oligoasthenozoospermic subjects.

	Control	LY294002 (10 μmol/l)
Percentage of forward motility	35.7 ± 5.1	49.8 ± 4.7[a]
Number of spermatozoa recovered after swim-up ($\times 10^6$)	13.3 ± 3.9	19.0 ± 4.8[b]
Sperm motility 2 h after swim-up	54.2 ± 4.5	68.6 ± 3.9[a]

[a]$P < 0.001$.
[b]$P < 0.005$ versus control.

(not shown). However, when the treatment was performed in the presence of LY294002, forward motility was preserved with both agents at concentrations similar or even greater than control (Figure 5A,B).

Following cryopreservation of seminal fluid, sperm motility has been shown to decrease due to damage of the cells during the processes of freezing and thawing (Royère *et al.*, 1996). In order to evaluate if LY294002 could have a protective effect on sperm motility during cryopreservation processes, semen samples from normospermic subjects were treated with the inhibitor before adding the cryoprotectant. One week after cryopreservation spermatozoa were thawed and motility evaluated. No differences were observed between LY294002-treated and control samples (C = 11.4 ± 6.4, LY 10 μmol/l = 11.6 ± 6.5, $n = 5$). To ascertain whether the addition of cryoprotectant could be responsible for lack of stimulatory effect of LY294002 on sperm motility, we tested the effect of the inhibitor in sperm samples after addition of the cryoprotectant. As shown in Figure 6, in the presence of cryoprotectant, the stimulatory effect of LY294002 was no
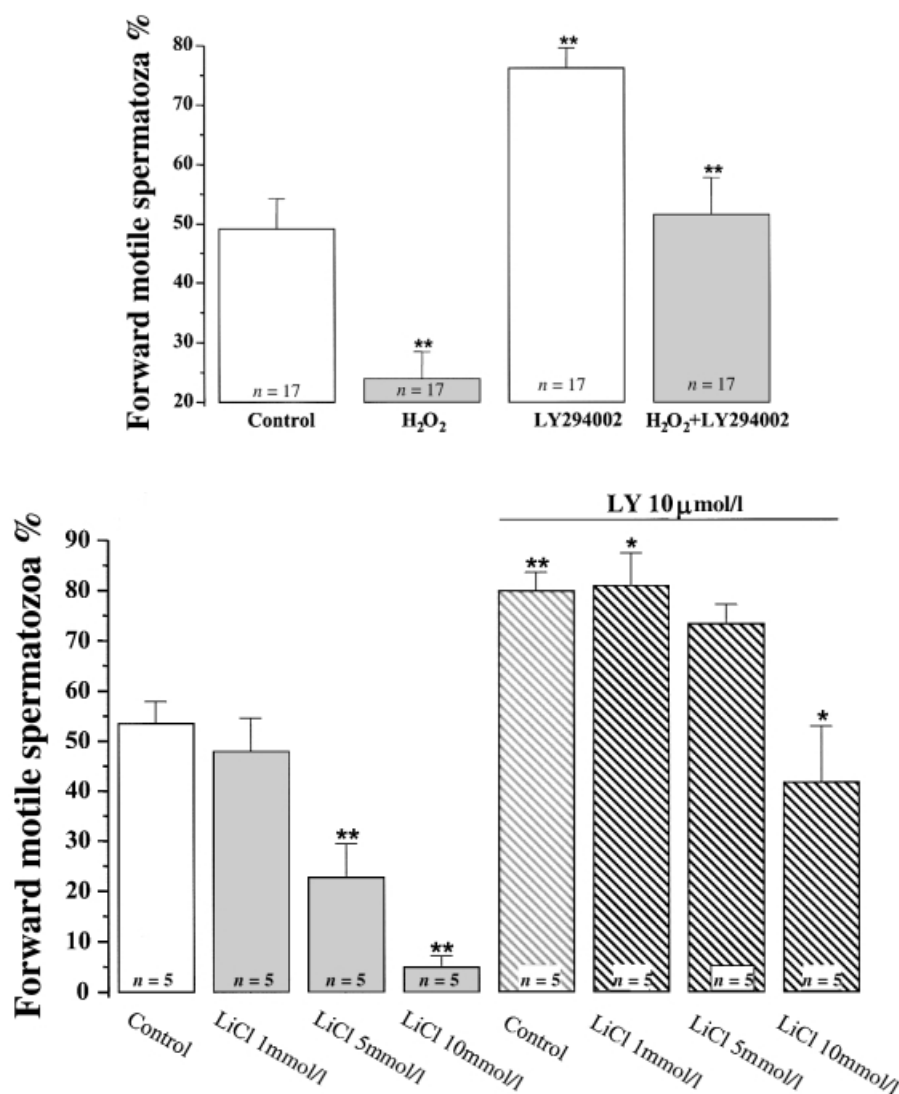
Figure 5. Effect of hydrogen peroxide (H_2O_2) (200 µmol/l, panel A) or LiCl (1–10 mmol/l, panel B) on forward motility of human spermatozoa in the presence or absence of LY294002 (LY) (10 µmol/l). The number of experiments is indicated inside the columns. *$P < 0.01$ or **$P < 0.0001$ versus respective control, t-test for paired data.

longer present, indicating that the fluid may contain something that does not allow the compound to penetrate spermatozoa or to interfere with its action.

To exclude the possibility that LY294002 could have toxic effects on spermatozoa, incubation with the two agents was prolonged for 24 h and sperm viability evaluated by hyposmotic swelling test. No decrease in sperm viability was observed with 100 µmol/l LY294002 (C = 82.0 ± 2.8, LY = 87.8 ± 4.1, $n = 6$).

Discussion

The present study demonstrates that inhibition of phosphatidylinositol 3-kinase (PI3K) is able to increase motility of human spermatozoa. Specifically, we show that addition of LY294002, an inhibitor of this lipid kinase, to swim-up selected spermatozoa or directly to seminal plasma increases forward motility, hyperactivation and the number of motile spermatozoa recovered after swim-up procedure. In addition, the inhibitor was also able to overcome the dramatic decrease of motility

observed after treatment with H_2O_2 and LiCl. Overall, our results imply a negative role for PI3K in development and maintaining of sperm motility, and suggest a possible use of PI3K inhibitors to increase motility in sperm samples prepared for assisted reproduction techniques. During the past few years, the higher fertilization success achieved with ICSI compared with traditional assisted reproduction techniques has enormously increased its application and many centres currently prefer this procedure to IVF for men with borderline sperm parameters. However, arguments such as the slight increase of sex chromosome abnormalities in the conceptus (Luetjens et al., 1999), the far more expensive costs of ICSI and the fact that it is more invasive than conventional IVF, still tend to maintain IVF as the first option. In this light, the possibility of recovering a higher number of spermatozoa showing better forward motility could allow several oligoasthenospermic men to enter IVF rather than ICSI programmes.

At variance with other stimuli able to increase sperm motility, such as platelet-activating factor (Krausz et al., 1994) and pentoxifylline (Tesarik et al., 1992), where the presence
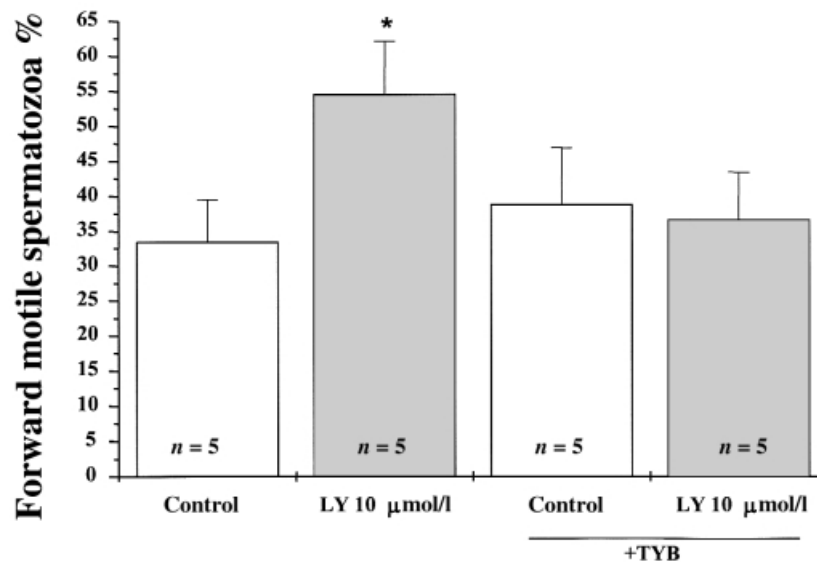
Figure 6. Effect of LY294002 (10 µmol/l) on sperm motility in the presence or absence of test yolk buffer (TYB) added to sperm suspension in five different experiments. *$P < 0.002$ versus control, *t*-test for paired data.

of non-responders has been described, LY294002 determined an increase of motility in spermatozoa from all the 116 subjects studied, suggesting the absence of non-responders. In addition, the drug did not affect sperm viability even after 24 h of treatment, indicating the absence of toxic effects. Furthermore, the inhibitor was able to increase VCL, VAP, VSL and HA as evaluated by CASA. However, despite the increase in percentage hyperactivated spermatozoa, ALH and beat-cross frequency (BCF) did not increase significantly due to the fact that the percentage of hyperactive cells is small. For this reason, although an increase in average values of BCF and ALH following treatment with the inhibitor was observed, this increase does not reach the statistical significance.

We also investigated whether LY294002 could be used to overcome the inhibitory effects on sperm motility following the process of cryopreservation. Unfortunately, one or more components of the test yolk buffer utilized for sperm cryopreservation in our study appear to prevent the stimulatory effect of LY294002 on sperm motility; this does not allow, for the moment, the possible evaluation of a protecting effect of this compound on sperm motility following cryopreservation. Further experiments are needed in order to determine which component(s) is responsible for such inhibitory effects.

The molecular mechanisms underlying the stimulatory effects of LY294002 on sperm motility are still obscure and are under investigation in our laboratory. The process of development and maintaining motility in mammalian spermatozoa is rather complex and involves the integration and crosstalk of several signalling pathways, including adenylate cyclase/cAMP/PKA, calcium and phosphorylation/dephosphorylation of proteins (Tash and Bracho, 1994). It is possible that, in spermatozoa, PI3K is involved in phosphorylating or dephosphorylating proteins that regulate motility. On the other hand, we cannot exclude that phosphoinositide phosphorylation, which is catalysed by PI3K, is also involved in the regulation of sperm motility. In somatic cells, PI3K is involved in the generation of reactive oxygen species (ROS) (Nishioka *et al.*,

1998) and ROS may activate PI3K (Shaw *et al.*, 1998). In view of the highly negative impact of high concentrations of ROS on sperm motility (Griveau and Le Lannou, 1997), it can be tentatively speculated that PI3K inhibition might reduce both ROS formation and/or ROS-mediated activation of PI3K. We show here that addition of LY 294002 indeed overcomes the decrease of motility determined by H_2O_2, suggesting a possible role for PI3K in mediating the detrimental effect of ROS on sperm motility. Similarly, reversion of the detrimental effect on motility after addition of LiCl suggests an involvement of PI3K in the action of this salt.

In conclusion, we present here consistent evidence that inhibition of PI3K increases forward motility in spermatozoa prepared for assisted fertilization techniques. Although further studies are needed to assess the safety of these compounds on the outcome of pregnancy, these results may have an important clinical impact, increasing the chance of undergoing IVF techniques in patients characterized by low sperm count and motility, and thus scheduled for ICSI .

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