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Human sperm motility is downregulated by the AMPK activator A769662

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SUMMARY

AMP-activated kinase (AMPK) plays a key function in maintaining cellular energy homeostasis. We recently identified and localized AMPK protein in human spermatozoa and showed that inhibition of AMPK activity significantly modified human sperm motility. Recently, AMPK has gained great relevance as prime target for pharmacological approaches in several energy-related pathologies and therefore pharmacological research is aimed to develop direct AMPK-activating compounds such as A769662. Our aim was to investigate the effect of A769662 in essential functional processes of human spermatozoa. Human spermatozoa were incubated in the presence or absence of the AMPK activator A769662 for different incubation times (0–20 h) and motility was evaluated by CASA system whereas other functional parameters were evaluated by flow cytometry. A769662 treatment significantly reduces the percentages of motile, progressive, and rapid spermatozoa starting at 2 h. Moreover, AMPK activator in human spermatozoa causes a significant reduction in any velocity measured, which is concomitant to a significant decrease in the percentage of rapid spermatozoa, both at short- (2–3 h) and long-time treatment (20 h). Treatment of human spermatozoa with A769662 does not significantly alter any of the following functional parameters: sperm viability, mitochondrial membrane potential, phosphatidylserine translocation to the outer leaf of plasma membrane, acrosome membrane integrity, or mitochondrial superoxide anion production. In summary, our results suggest that AMPK in human spermatozoa contributes to the regulation of sperm motility, without affecting basic physiological parameters of human spermatozoa (viability, mitochondrial membrane potential or reactive oxygen species production, acrosome membrane integrity, phosphatidylserine exposure at plasma membrane). As sperm motility is required in the female reproductive tract to achieve fertilization, we conclude that AMPK is an essential regulatory kinase of the human spermatozoa function. This conclusion needs to be taken into account when AMPK is elected as prime target in pharmacological approaches for several energy-related pathologies.

INTRODUCTION

Fertilization is achieved once spermatozoa have acquired a fertilizing competent status conferred by different processes occurring within female reproductive tract, including motility, hyperactivation, and capacitation. Achieving of these functional competences in spermatozoa is primary due to phosphorylation of proteins present in mature sperm cells and is greatly dependent on the cellular energy charge, that is, the ratio between cellular ATP and AMP levels (Garrett *et al.*, 2008). The AMP-activated protein kinase AMPK has emerged as a major regulator of cellular energy homeostasis (Carling *et al.*, 2012; Hardie *et al.*, 2016). AMPK is an evolutionary conserved serine/threonine kinase that becomes activated under ATP-limiting conditions to coordinate a wide array of energy-sparing responses by inhibiting ATP-consuming anabolic pathways and stimulating ATP-generating pathways (Carling *et al.*, 2012; Hardie *et al.*, 2016). AMPK is a heterotrimeric protein composed by three subunits: catalytic α, scaffolding β, and regulatory γ. This last subunit

contains two Bateman domains that bind its positive allosteric effector AMP (Scott *et al.*, 2004). AMPK is highly sensitive to AMP, as any increase in the ratio AMP/ATP caused by a decrease in cellular energy state, stimulates AMPK enzymatic activity by increasing AMPK phosphorylation at Thr-172 located in the activation loop of α subunit (Suter *et al.*, 2006). Among the kinases described to phosphorylate AMPK at Thr172 in somatic cells are as follows: (i) the tumor suppressor kinase accountable for the inherited cancer disorder Peutz–Jeghers syndrome, LKB1 (Woods *et al.*, 2003), (ii) the two Ca^{2+}/calmodulin-dependent protein kinase kinases CaMKKα and CaMKKβ (Hawley *et al.*, 2005), and (iii) the transforming growth factor TGF-β-activated kinase-1, TAK1 (Xie *et al.*, 2006). Overall, AMPK by integrating multiple extra- and intracellular stimuli including different types of cellular and metabolic stresses (Evans *et al.*, 2005; Long & Zierath, 2006) can regulate cell processes outside metabolism (Carling *et al.*, 2012; Hardie *et al.*, 2016).

The first evidence of AMPK protein expression in mature mammalian spermatozoa was described by our group in 2012 (Hurtado de Llera *et al.*, 2012). AMPK is mainly localized in the acrosome region of sperm head and in the flagellum midpiece of porcine spermatozoa (Hurtado de Llera *et al.*, 2013). Detailed study of the AMPK function demonstrated that is essentially involved in the regulation of one of the most important sperm functions, motility (Hurtado de Llera *et al.*, 2012), as well as in regulatory adjustments in the mitochondrial membrane potential, sperm plasma membrane fluidity and organization, and acrosome integrity at 37 °C (Hurtado de Llera *et al.*, 2013) and also during boar semen preservation at 17 °C (Martin-Hidalgo *et al.*, 2013).

Our group has recently identified the AMPK protein in human spermatozoa where is localized at the sperm head, particularly at the acrosome, the midpiece and along the sperm tail (Calle-Guisado *et al.*, 2016). Supporting an important function of AMPK in the sperm physiology (Tartarin *et al.*, 2012) showed that mice lacking the AMPK catalytic subunit α1 gene [α1AMPKα1knockout (KO)] exhibit a reduced fertility accompanied by an alteration in sperm morphology, decreased mitochondrial membrane potential, lower basal oxygen consumption as well as decreased sperm motility. Among stimuli leading to AMPK activity in boar spermatozoa are external Ca^{2+} and HCO_3^- that through the activation of soluble adenylyl cyclase produce an increase in intracellular cAMP, and also different types of sperm stresses (Hurtado de Llera *et al.*, 2014). Boar sperm kinases involved in AMPK signaling pathway are protein kinase A (PKA) that results an essential protein for sperm AMPK activity, CaMKKα/β, and PKC (Hurtado de Llera *et al.*, 2015, 2016).

On account of its key function in maintaining cellular energy homeostasis, AMPK has recently achieved great relevance as prime target in pharmacological approaches for several cell energy-related pathologies such as type 2 diabetes, insulin resistance, cardiovascular diseases, and/or cancer. Moreover, AMPK has also been proposed as a promising therapeutic target to treat neurodegenerative diseases such as Huntington disease (Walter *et al.*, 2016) or to treat neuropathic pain (Price & Dussor, 2013). The number of increasing studies in which AMPK is pointed as a key kinase in the course of different disorders has led to focus clinical research on the development of direct AMPK-activating compounds. The first reported small-molecule direct-specific activator of AMPK was the thienopyridone drug A769662 (Cool *et al.*, 2006) that activates AMPK both allosterically and by inhibiting the dephosphorylation of AMPK in Thr172 (Cool *et al.*, 2006; Sanders *et al.*, 2007). Several studies have fairly established that A769662 selectively binds and activates AMPK (Goransson *et al.*, 2007; Sanders *et al.*, 2007; Scott *et al.*, 2008; Rajamohan *et al.*, 2016) independently of the upstream kinase involved (Goransson *et al.*, 2007). A769662 effects have been studied in different somatic cell types, such as STHdh immortalized striatal progenitor cells (Walter *et al.*, 2016), heart and lung cells (Rameshrad *et al.*, 2016). As mentioned, spermatozoa need to adapt to external changing conditions as well as metabolic or environmental stresses that might physiologically occur during their transit through the female reproductive tract. Our hypothesis is that these necessary adaptations of human spermatozoa metabolism to environmental changes would definitely be controlled by AMPK activity fluctuations, either up or down, as it occurs in boar spermatozoa (Hurtado de Llera *et al.*, 2015). As the unique study of AMPK in human spermatozoa has been performed by studying down fluctuation—inhibition—of AMPK (Calle-Guisado *et al.*, 2016), this work aims to study the role of the AMPK activator A769662 in those main functional processes of human spermatozoa that are required to successfully accomplish the oocyte fertilization. The functional consequences of an enhanced AMPK activity in human spermatozoa would be essential for the understanding of those sperm processes in which the energy sensor AMPK is likely involved and therefore should be taken into account when AMPK activators (i.e., by A769662) are selected as a key target for pharmacological approaches in different cell energy-related pathologies.

MATERIALS AND METHODS

Chemicals and sources

The sperm density gradient Spermfilter® was from Gynotec (The Netherlands, EU) and SWM was from IrvineScientific (Daimler, St. Santa Ana, CA, USA). A769662 was from Tocris Bioscience (Bristol, UK); live/dead spermatozoa viability kit (including both propidium iodide (PI) and SYBR-14 probes), MitoSOX™ Red, and YoPro-1 probes were from Molecular Probes (Leiden, The Netherlands); PNA-FITC was from Sigma-Aldrich® (St Louis, MO, USA); annexin-V–FITC from Immunostep (Salamanca, Spain); JC-1 probes from Life Technologies Ltd (Grand Island, NY, USA); coulter isotone II diluent from Beckman Coulter Inc. (Brea, CA, USA).

Ethical approval

The study was conducted in accordance with ethical guidelines, and informed and written consent was obtained from all individuals included in the study. Human semen was obtained from healthy donors, prepared, and evaluated in line with the recommendations and current values of the World Health Organization (WHO). The University of Extremadura Ethical Committee approved all protocols.

Human semen samples

Semen samples from nine healthy donors were obtained by masturbation into a sterile plastic container after 2–3 days of sexual abstinence, in line with the recommendations of the World Health Organization (WHO), and analyzed following it. After complete liquefaction (between 10 min and 1 h at 37 °C),

samples were examined and processed. The semen parameters (total fluid volume, sperm concentration, motility, and morphology) of all the samples fell within the WHO normality criteria. Two different fractions of spermatozoa (high and low motility) were separated by Spermfilter®, a silane-silica based 40–80% density gradient medium used in artificial reproduction techniques for the separation and purification of highly motile human spermatozoa. Briefly, aliquots of semen (1 mL) from nine donors (n = 9) were layered over the upper layer of the density gradient and centrifuged at room temperature for 20 min at 300 ***g***. Two sperm fractions were collected: high- and low-motility fractions that were diluted in sperm-washing medium SWM, that includes sodium chloride, potassium chloride, magnesium sulfate anhydrous, potassium phosphate monobasic, calcium chloride anhydrous, glucose, sodium pyruvate, sodium lactate, phenol red (a pH indicator), sodium bicarbonate, 4-(2- hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and human serum albumin (HSA). Sperm concentration and motility parameters were evaluated in each sperm fraction.

Human sperm motility analysis

High-motility human sperm fractions from nine individuals donors with normal sperm parameters according to WHO were incubated at 20 × 10^6 cells/mL in SWM with an equal volume of the AMPK activator A769662 (100 μM) or the solvent dimethyl sulfoxide (DMSO) (0.024%) for different times (0, 1, 2, 3, and 20 h) in a humidified atmosphere of 5% CO_2 in air at 37 °C. Then, 6 μL of sample was placed in a pre-warmed Spermtrack sperm-counting chamber where following motility parameters were evaluated using the ISAS system (PROISER, Paterna, Valencia, Spain): percentage of total motile, rapid and progressive spermatozoa, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head movement (ALH), linearity (LIN), and beat cross of flagellum frequency (BCF). The settings of the system analyzer were as follows: frame rate, 60 Hz; frame acquired, 25; straightness threshold, 80%; temperature 37 °C. The total number of spermatozoa evaluated in each semen sample was at least 300.

Analysis of human spermatozoa viability by flow cytometry

Fluorescent staining using the live/dead sperm viability kit was performed to measure sperm viability. Briefly, 200 μL of semen sample (2 × 10^6 cells per mL) were incubated in SWM for 20 min at room temperature in darkness with 2.5 μL of SYBR-14® dye (2 μM) and 5 μL of propidium iodide (PI) (480 μM), followed by an analysis in the flow cytometer ACEA NovoCyte TM (ACEA Biosciences, Inc., San Diego, CA, USA) using ACEA NOVOEXPRESS TM software. The fluorescence values of SYBR-14 were collected in the laser-excited fluorescence channel (BL1) using a 525-nm band-pass filter, whereas PI fluorescence was collected in the BL3 channel using a 620-nm band-pass filter. The results of viable spermatozoa were expressed as the average of the percentage of SYBR-14^+ and PI^- labeled cells ± standard error of the mean.

Analysis of human spermatozoa acrosome integrity by flow cytometry

The population of spermatozoa with reacted or damaged acrosome was assessed after staining these germ cells with fluorescein isothiocyanate-conjugated peanut agglutinin (PNA-FITC) as a specific marker for acrosome membrane status and PI as a marker for cell death. Aliquots of 100 μL of each semen sample (2 × 10^6 cells/mL) were incubated at RT in darkness for not more than 5 min with 5 μL of PNA-FITC (25 μg/mL) and 5 μL of PI (240 μM). Then, 150 μL of SWM was added to each sample and mixed before flow cytometry analysis. The fluorescence value of probe PNA-FITC was collected in the channel BL1 using a 525-nm band-pass filter. Results are expressed as the average of the percentage of PNA^+ and PI^- spermatozoa ± standard error of the mean.

Analysis of human sperm mitochondrial membrane potential (ΔΨm) by flow cytometry

Mitochondrial membrane potential variations (ΔΨm) were evaluated using the specific probe JC-1 (5,5′,6,6′–tetrachloro-1,1′,3,3′ tetraethylbenzymidazolyl carbocyanine iodine). This lipophilic cationic fluorochrome is present as protomeric aggregates in mitochondria with high membrane potential that emit in orange (590 nm), whereas in mitochondria with low membrane potential, JC-1 is present as monomers that emit in green (525 nm), when is excited at 488 nm.

Briefly, an aliquot of 200 μL from each sperm sample (2 × 10^6 cells/mL) was incubated with 20 μL of JC-1 (60 μM) and then mixed and incubated at 37 °C for 1 h. The samples were mixed again before flow cytometry analysis. The fluorescence values of probe JC-1 were collected in the channel BL1 using a 525-nm band-pass filter and in the channel BL2 using a 585-nm band-pass filter. The percentage of orange-stained cells was recorded and considered the population of spermatozoa with a high mitochondrial membrane potential. There is a third population that can be called medium mitochondrial membrane potential. Results are expressed as the average percentage of orange-stained (high ΔΨm) spermatozoa ± standard error of the mean.

Analysis of human sperm phosphatidylserine externalization at the outer leaflet plasma membrane by flow cytometry

The study of phosphatidylserine (PS) externalization in plasma membrane spermatozoa was performed using annexin-V–FITC to specifically detect PS translocation from the inner to the outer leaflet of the human sperm plasma membrane. Briefly, 15 μL (20 × 10^6 cells/mL) sperm cells were diluted in 100 μL of the following buffer: 96 mmol/L NaCl, 4.7 mmol/L KCl, 0.4 mmol/L $MgSO_4$, 0.3 mmol/L NaH_2PO_4, 5.5 mmol/L glucose, 1 mmol/L sodium pyruvate, 21.6 mmol/L sodium lactate, 20 mmol/L HEPES (pH 7.45), and 2.5 mmol/L $CaCl_2$, followed by incubation at 37 °C with 5 μL of annexin-V-FITC and 5 μL of IP (480 μM). Finally, 100 μL of SWM was added to each sample and mixed before flow cytometry analysis. The fluorescence values of probes annexin V-FITC and PI were collected in the BL1 channel and BL3 channel using a 520- and 620-nm band-pass filter, respectively. The results are expressed as the average of the percentage of annexin V^+/PI^- spermatozoa ± standard error of the mean.

Analysis of mitochondrial reactive oxygen species production in human sperm by flow cytometry

MitoSOX™ Red was used to measure mitochondrial reactive oxygen species (ROS) production. MitoSOX™ Red reagent enters the mitochondria and is oxidized by the superoxide anion. The resulting oxidation product becomes highly fluorescent upon binding to nucleic acids. Briefly, an aliquot of 250 μL from each

sperm sample (2×10^6 cells/mL) was incubated with 0.5 μL of MitoSOX™ (2 μM), mixed, and incubated at 37 °C for 15 min. The samples were mixed again before flow cytometry analysis, and the fluorescence values of MitoSOX™ were collected in the BL2 channel using a 585-nm band-pass filter. Results are expressed as the mean percentage of MitoSOX-positive sperm ± standard error of the mean.

Statistical analysis

The program IBM SPSS 19 (Armonk, NY, USA) has been used to perform the statistical analysis. Two-way repeated measures ANOVAS have been used to compare the mean differences between groups that have been split into two within-subject factors (treatment and time). Interactions and principal effects were analyzed. Applicability conditions were directly met or, in the cases where any assumption was violated, alternative solutions were obtained to overcome this problem. Results were considered significant when p values were lower than 0.05.

RESULTS

The AMPK activator A769662 causes a reduction in human sperm motility

We have recently demonstrated that A769662 also works as a potent activator of AMPK in mammalian sperm cells (Hurtado de Llera *et al.*, 2015) in a time and concentration dependent manner, being 100 μM the concentration with clear effect. To evaluate the effect of an increase in the physiological levels of AMPK activity in human sperm motility, cells were incubated in the absence (DMSO) or presence of A769662 (100 μM) for different times (1, 2, 3, and 20 h) at physiological temperature, 37 °C. As observed in Fig. 1A, enhanced AMPK activity leads to a time-dependent reduction in the percentage of motile spermatozoa, being statistically significant at 2 h and remaining until 20 h. Among motile spermatozoa, those with progressive motility play special relevance. As observed in Fig. 1B, an increase in AMPK activity leads to a clear and rapid reduction in this parameter, which is statistically significant at 2 h and 3 h and maximal at 20 h (by 50% reduction). Regarding the percentage of rapid spermatozoa, defined as those motile spermatozoa with average velocity VAP higher than 35 μm/sec (Fig. 1C), an enhanced AMPK activity causes a significant reduction in this motility parameter, expressed as the percentage of motile spermatozoa. This effect of A769662 is maximal at 20-h incubation where the percentage of rapid spermatozoa decreased by 50%. It is worth to notice that the longest incubation time studied (20 h) is not the optimal experimental condition to study rapid human spermatozoa, as there is a 50% decrease in this parameter (from 63 to 31% of motile spermatozoa) in control conditions (in the absence of A769662, Fig. 1C, white histograms).

The analysis of human sperm velocities reveals that the curvilinear velocity VCL (Fig. 2A), the straight-line velocity VSL (Fig. 2B), and the average velocity VAP (Fig. 2C) are always significantly reduced in the presence of the AMPK activator at any time studied, reaching a maximal effect at 20 h. Moreover, enhanced AMPK activity induced by A769662 in human spermatozoa leads to a significantly reduction in other spermatozoa motility parameters analyzed the following: LIN, STR, WOB, ALH, and BCF (Table 1). LIN and STR parameters present a significant reduction at 2 and 3 h and maximal at 20 h, WOB at 3 and 20 h, and BCF and ALH parameters at any time studied. Furthermore, the interaction between incubation time and treatment is statistically significant.

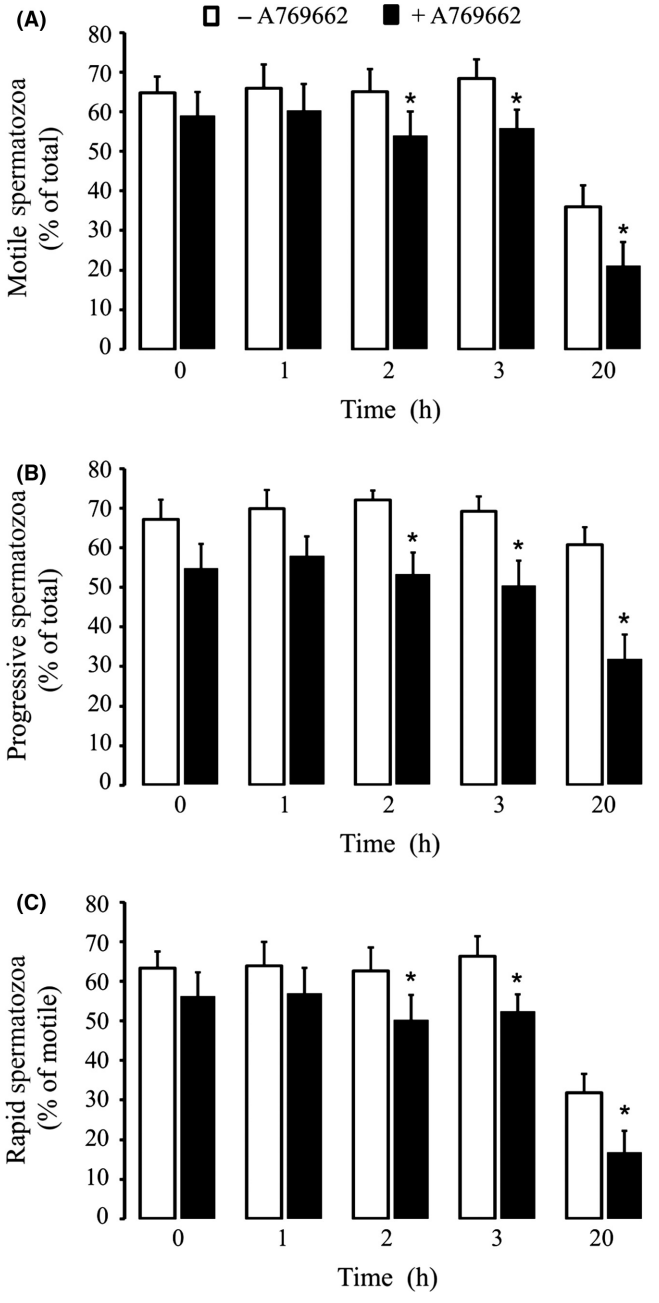
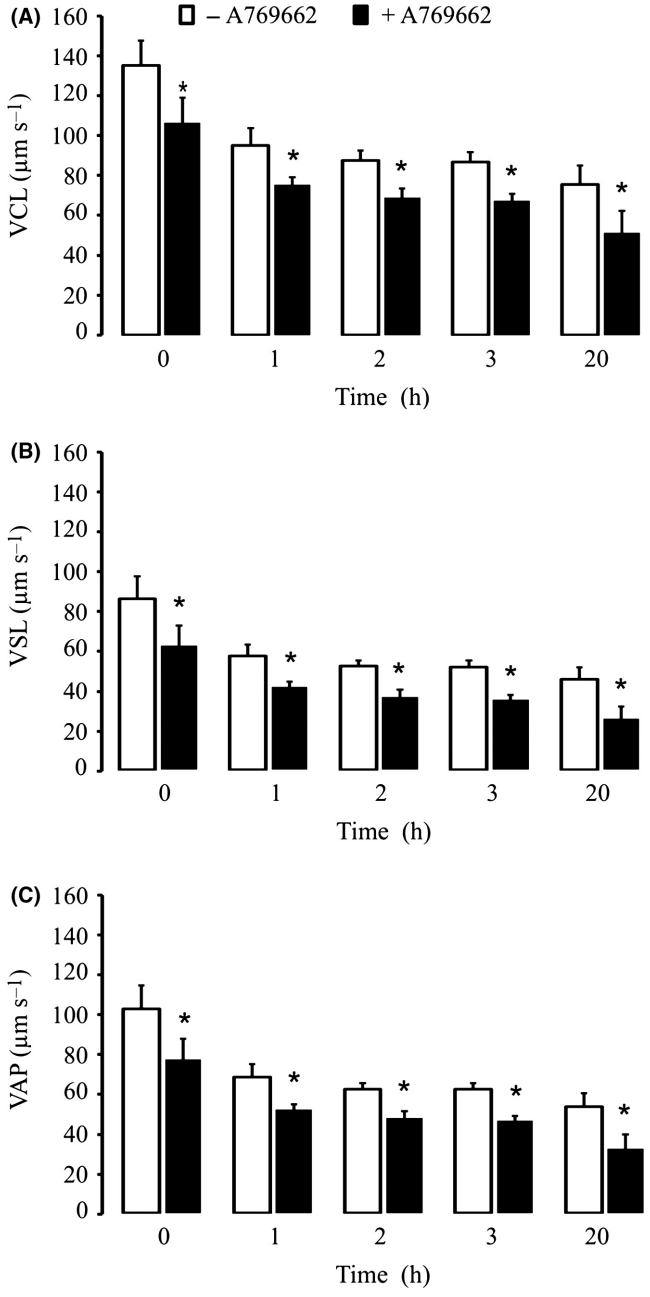


Figure 1 Time-course effect of AMPK activator A769662 in the percentages of motile (A), rapid (B), and progressive (C) human spermatozoa. After isolation by density gradient, human spermatozoa were incubated at 37 °C in the absence (DMSO, white histograms) or presence (black histograms) of AMPK activator A769662 (100 μM) for indicated times (0–20 h). Following sperm motility parameters were evaluated by ISAS system: percentages of motile spermatozoa (Fig. 1A), those motile spermatozoa with progressive motility, that is, a+b (1B) and rapid spermatozoa (1C). This experiment was performed at least nine times, and results express the mean of the percentages of total sperm ± standard error of the mean. Statistical differences were considered when $p < 0.05$ and showed with an asterisk.

Effect of A769662 in human sperm viability

The effect of A769662 in human sperm viability has been analyzed to correlate it with motility and other functional studies,

Figure 2 Time-course effect of A769662 in human spermatozoa velocities: curvilinear VCL (A), straight-line VSL (B), and average path VAP (C). After isolation by density gradient, human spermatozoa were incubated at 37 °C in the absence (DMSO, white histograms) or presence (black histograms) of AMPK activator A769662 (100 μM) for indicated times (0–20 h). Following sperm motility parameters were evaluated by ISAS system: curvilinear velocity VCL (Fig. 2A), straight-line velocity VSL (2B), and average velocity VAP (2C), expressed as μm/sec. This experiment was performed at least nine times, and results express the mean of the percentages of total sperm ± standard error of the mean. Statistical differences were considered when $p < 0.05$ and showed with an asterisk.



and in addition to know whether an increase over the physiological AMPK activity induced by A769662 might cause human sperm side effects that compromise cell viability. Results in Fig. 3 show that human sperm viability, measured as percentage of SYBR-14$^+$/IP$^-$ spermatozoa, remains constant at any short time analyzed, about 82% of total spermatozoa are viable (Fig. 3, white histograms), and is not affected by an enhanced AMPK activity due to A769662 (Fig. 3). Longer incubation time in control conditions (absence of A769662) causes about 25% reduction in human sperm viability (Fig. 3, white histograms 20 h), which is not affected by A769662.

Effect of A769662 in the mitochondrial membrane potential of human spermatozoa

The effect of enhanced AMPK activity in the sperm mitochondrial membrane potential, ΔΨm, was evaluated after incubation of human spermatozoa in the presence or absence of A769662 (100 μM) for different times at 37 °C (Fig. 4). An increased activity of AMPK induced by A769662 does not modify the population of human spermatozoa presenting high ΔΨm at any time studied except at 3 h where a slight increase but no significant A769662 effect of this parameter is observed (Fig. 4).

Effect of A769662 in the mitochondrial superoxide anion production of human spermatozoa

The effect of A769662 in the mitochondrial ROS was evaluated after incubation of human spermatozoa in the presence or absence of A769662 (100 μM) for different times at 37 °C (Fig. 5). Treatment with A769662 does not modify the population of human spermatozoa with high mitochondrial ROS levels at any time studied (Fig. 5).

Effect of A769662 in the phosphatidylserine externalization in human sperm plasma membrane

We investigated the effect of an increased AMPK activity in the phosphatidylserine (PS) translocation to the outer leaf of human sperm plasma membrane, process that might indicate plasma membrane scrambling (Gadella & Harrison, 2000), which physiologically occurs in relevant sperm functional processes. As observed in Fig. 6, the levels of phosphatidylserine externalized in human sperm plasma membrane in the absence of A769662 (white histograms) are very low (between 4–7% of total sperm) during short times at physiological temperature 37 °C and slightly increases at 20 h (11% of total). Under these experimental conditions, a sustained increase in AMPK activity does not significantly modify the degree of phosphatidylserine externalization.

Effect of A769662 in the acrosome membrane integrity of human spermatozoa

We next analyzed the effect of an increase in AMPK activity on the acrosome membrane integrity of human spermatozoa. Spermatozoa labeling with PNA is indicative of the integrity of acrosome membrane and might reflect both damaged as reacted acrosome. Acrosome membrane integrity is generally maintained in human spermatozoa under control conditions over the time, as observed in Fig. 7, where it is observed that more than 99% of live spermatozoa PI$^-$ are PNA$^-$ (white histograms). The human sperm acrosome integrity is not significantly affected by enhanced AMPK activity, although A769662 causes a slight increase in the population of human spermatozoa PNA$^+$ at time 0 (from 0.66 to 1.52% in the absence and presence A769662, respectively) and at 20 h (from 0.62 to 1.63% spermatozoa in the absence and presence of A769662, respectively).

DISCUSSION

Sperm cells achieve a fine regulation of metabolism by dynamic molecular mechanisms that effectively manage their

Figure 3 Time-course effect of A769662 in human sperm viability. After isolation by density gradient, human spermatozoa were incubated at 37 °C in the absence (DMSO, white histograms) or presence (black histograms) of AMPK activator A769662 (100 μM) for indicated times (0–20 h). Sperm viability was measured by flow cytometry using SYBR-14 and IP as probes. Representative two-dimensional SYBR14 fluorescence vs. PI fluorescence dot plots for sperm samples incubated in the presence or absence of A769662 for the maximum time (20 h) are shown at the right side. This experiment was performed five times and results, which are expressed as percentage of total sperm cells analyzed, are shown as mean ± standard error of the mean. Statistically significant differences were not found with $p < 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com].

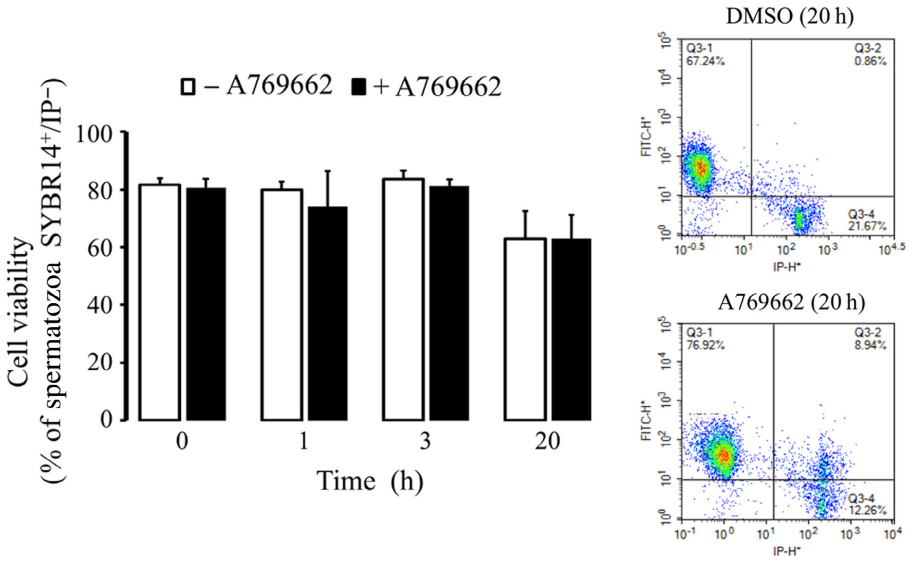
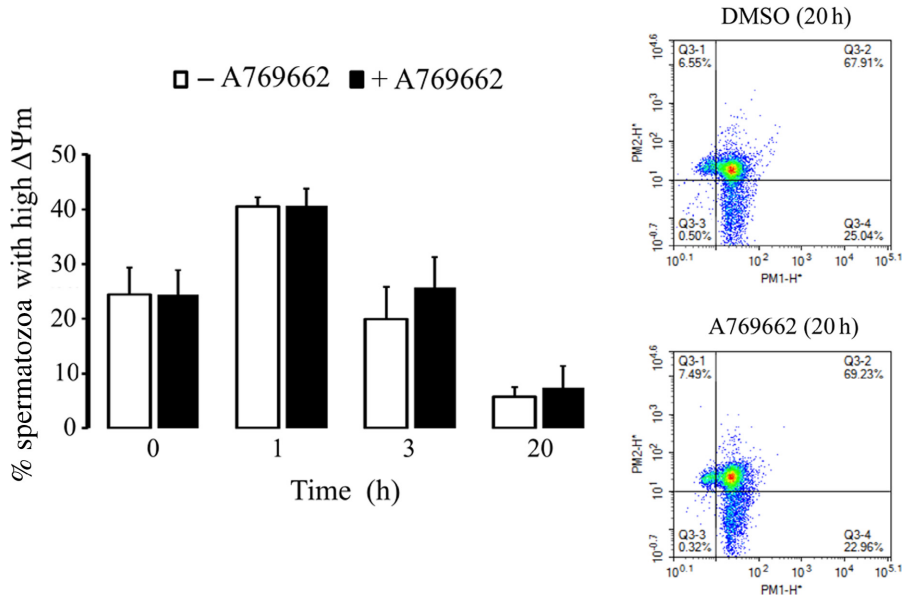


Figure 4 Time-course effect of A769662 in the mitochondrial membrane potential of human spermatozoa. After isolation by density gradient, human spermatozoa were incubated at 37 °C in the absence (DMSO, white histograms) or presence (black histograms) of AMPK activator A769662 (100 μM) for indicated times (0–20 h). Mitochondrial membrane potential was measured by flow cytometry using JC-1 as a probe. Sperm population exhibiting high ΔΨm are expressed as percentage of total sperm cells analyzed. Representative two-dimensional fluorescence dot plots for sperm samples incubated in the presence or absence of A769662 for the maximum time (20 h) are shown at the right side. Each experiment was performed at least five times, and results express the mean ± standard error of the mean. Statistically significant differences were not found with $p < 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com].



adjustment to those changes in extracellular medium that physiologically occur in the female reproductive tract. The control of energy status during fluctuating extracellular conditions that accompany fertilization is fundamental for the knowledge of sperm function. Among the molecular mechanisms that sense cell energy and redox status is AMP-activating kinase, AMPK, a relevant kinase that has recently emerged as a key regulator of mammalian spermatozoa function in response to different stimuli including (Hurtado de Llera *et al.*, 2012, 2013, 2014, 2015, 2016). We have recently demonstrated that A769662 effectively enhances AMPK activity by increasing phosphorylation at Thr172 in boar spermatozoa in a time and concentration dependent manner (Hurtado de Llera *et al.*, 2015). Based on these experiments, we have selected 100 μM as the concentration of

Figure 5 Time-course effect of A769662 in the mitochondrial superoxide anion production of human spermatozoa. After isolation by density gradient, human spermatozoa were incubated at 37 °C in the absence (DMSO, white histograms) or presence (black histograms) of AMPK activator A769662 (100 μM) for indicated times (0–20 h). Mitochondrial ROS production was measured by flow cytometry using MitoSOX™ Red as a probe. MitoSOX-positive sperm population is expressed as percentage of total sperm cells analyzed. Representative two-dimensional fluorescence dot plots for sperm samples incubated in the presence or absence of A769662 for the maximum time (20 h) are shown at the right side. This experiment was performed at least five times, and results express the mean ± standard error of the mean. Statistically significant differences were not found with $p < 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com].

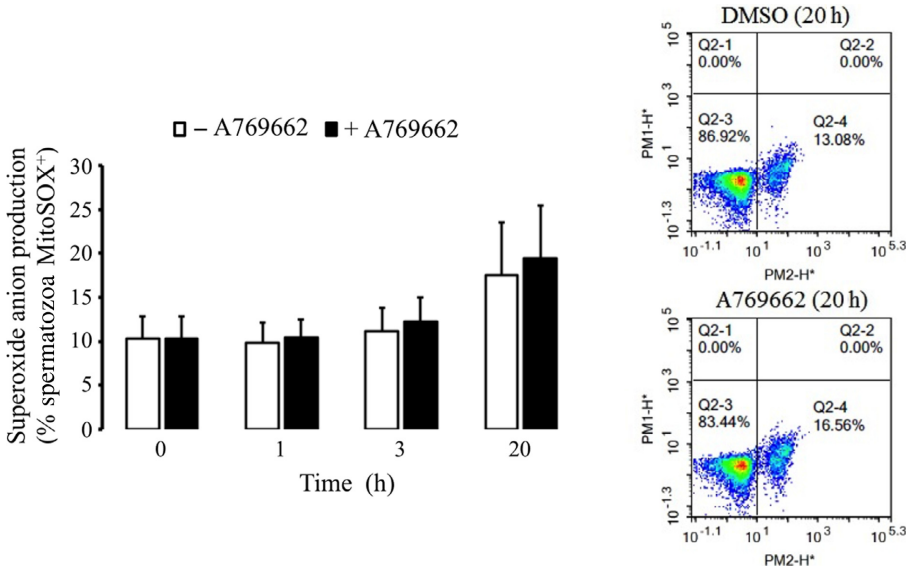
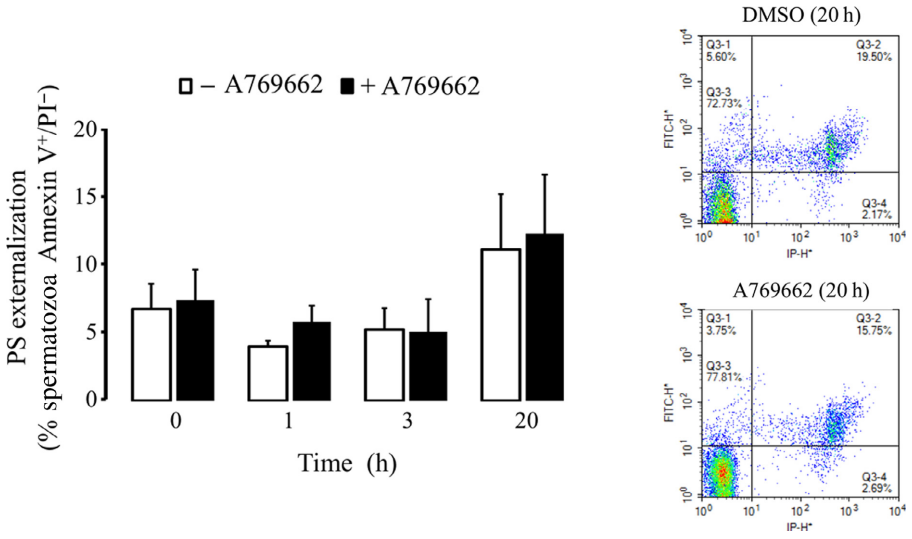
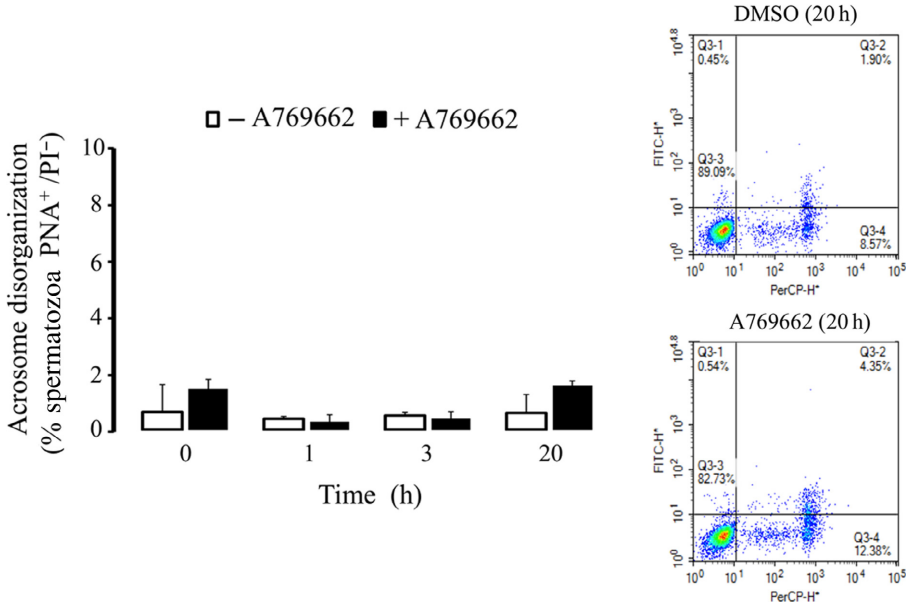


Figure 6 Time-course effect of A769662 in plasma membrane phosphatidylserine externalization of human spermatozoa. After isolation by density gradient, human spermatozoa were incubated at 37 °C in the absence (DMSO, white histograms) or presence (black histograms) of AMPK activator A769662 (100 μM) for indicated times (0–20 h). The level of phosphatidylserine (PS) externalization at the sperm plasma membrane was measured by flow cytometry using annexin V-FITC as probe. Sperm population exhibiting outward translocation of phosphatidylserine at the plasma membrane (annexin V^+) is expressed as the percentage of total live spermatozoa (PI^-) analyzed. Representative two-dimensional annexin-V fluorescence vs. PI fluorescence dot plots for sperm samples incubated in the presence or absence of A769662 for the maximum time (20 h) are shown at the right side. Each experiment was performed at least five times and the results express the mean ± standard error of the mean. Statistically significant differences were not found with $p < 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com].



A769662 to be used in human spermatozoa, which agrees with the concentration range that has also been described in different types of somatic cells (Ikematsu *et al.*, 2011; Walter *et al.*, 2016). In the present study, we show that treatment of human spermatozoa with AMPK activator A769662 causes a marked decrease in the number of motile spermatozoa along with a clear reduction in sperm velocities, in agreement with the inhibitory effect of A769662 in sperm motility from other mammalian species (Hurtado de Llera *et al.*, 2015). Importantly, under A769662 treatment, those remnant human spermatozoa that are still motile present a clearly less progressive movement together with a less efficient or competent motility. Thus, these data demonstrate

Figure 7 Time-course effect of A769662 in the acrosome membrane integrity of human spermatozoa. After isolation by density gradient, human spermatozoa were incubated at 37 °C in the absence (DMSO, white histograms) or presence (black histograms) of AMPK activator A769662 (100 μM) for indicated times (0–20 h). Destabilization of human acrosome membrane was measured by flow cytometry using PNA-FITC as a probe. Sperm population with unstable acrosomes (lower acrosome integrity) exhibiting PNA^+ labeling is expressed as percentage of total live (PI^-) spermatozoa analyzed. Representative two-dimensional PNA-FITC fluorescence vs. PI fluorescence dot plots for sperm samples incubated in the presence or absence of A769662 for the maximum time (20 h) are shown at the right side. Each experiment was performed at least five times and the results express the mean ± standard error of the mean. Statistically significant differences were not found with $p < 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com].



that the AMPK activator leads to a drastic reduction in human spermatozoa motility. However, we have recently described that AMPK inhibition in human spermatozoa for 20 h also causes an adverse effect in motility (Calle-Guisado *et al.*, 2016). Currently, with the existing data about AMPK and spermatozoa, we do not know the scientific explanation of these apparently conflicting data in human spermatozoa (either down or up-AMPK activity negatively affects motility) that, on the other hand, are in total agreement with the AMPK effects in boar sperm motility (Hurtado de Llera *et al.*, 2015). Keeping in mind its cellular energy-regulating role, it is logical that AMPK signaling is effectively involved in the regulation of motility, sperm function that is particularly dependent on the energy levels. In fact, this and our previous works allow us to propose that a proper level of AMPK activity (i.e., basal or situated within the sperm physiological range) and kept for an opportune physiological time is essential to maintain correct human sperm motility, which is adapted to the changing extracellular environment. This conclusion is supported by several studies in sperm cells where AMPK activity was experimentally modified out of physiological range in sperm cells for prolonged time. Thus, our group and others have demonstrated that permanent downregulation of AMPK activity below physiological levels using AMPK inhibitor compound C plays a negative role in sperm motility in human (Calle-Guisado *et al.*, 2016), boar (Hurtado de Llera *et al.*, 2012; Martin-Hidalgo *et al.*, 2013), chicken (Nguyen *et al.*, 2014), and mice (Tartarin *et al.*, 2012), this later work used a different approach with α1AMPK knockout mice. By contrary, the unique work studying an upregulation of AMPK activity above physiological levels for prolonged time in spermatozoa also describes an adverse effect in boar sperm motility (Hurtado de Llera *et al.*, 2015). A possible explanation for the fact that treatment with AMPK activator for prolonged time negatively affects human sperm motility is based on the previous proposal that a permanent increase in AMPK activity above physiological levels would lead to a chronic deregulation of sperm metabolism caused by a prolonged stimulation of ATP-generating catabolic pathways and by a permanent inhibition of ATP-consuming anabolic pathways (Hurtado de Llera *et al.*, 2015, 2016). This chronic disarrangement of human sperm metabolic homeostasis is not adequate at all for the maintenance of a correct sperm motility adapted to any extracellular conditions.

The above-discussed AMPK role in human sperm motility occurs without changes in the following human sperm processes analyzed in the present study: sperm viability, mitochondrial membrane potential, mitochondrial reactive oxygen species production, phosphatidylserine translocation, or acrosome membrane integrity. The lack of A769662 effect in these function-related processes in human spermatozoa contrasts with results previously described in boar spermatozoa (Hurtado de Llera *et al.*, 2015). In this later species, A769662 treatment caused some effects in sperm viability, ΔΨm, plasma membrane organization, and acrosome membrane integrity, which are always related or dependent on extracellular medium stimuli. The lack of effect of A769662 in mitochondrial reactive oxygen species production in human spermatozoa cannot be compared with other species, as this is the first work that addresses this point. In somatic cells, A769662 increased the expression of manganese superoxide dismutase, an essential antioxidant mitochondrial enzyme (Dang *et al.*, 2015). Accordingly, AMPK activation by metformin decreases ROS production in cardiomyocytes (Barreto-Torres *et al.*, 2015), confirming a beneficial action of AMPK activity against mitochondrial oxidative stress in somatic cells. In human spermatozoa, we have not found an effect of A769662

in the mitochondrial ROS levels, which can be explained by different reasons: (i) we have studied a completely different cell type which is no-somatic cell: spermatozoa; (ii) the described effect of AMPK on mitochondrial ROS production (Barreto-Torres *et al.*, 2015) was evaluated when AMPK was activated by metformin, which is an indirect AMPK activator exerting its action through the mitochondria, whereas A769662 is a specific, reversible, and direct AMPK activator that displays selectivity toward its β1 subunit-containing heterotrimers; (iii) the described action of AMPK in the increased expression of an antioxidant enzyme can be scientifically argued in somatic cells (Dang *et al.*, 2015) but not in spermatozoa, cell type that when mature lacks of transcription and translation.

Despite it has been described a toxic effect of 300 μM of A769662 increasing the number of non-viable MEF cells (Moreno *et al.*, 2008), we do not observe any toxic or deleterious effect of 100 μM A769662 in human spermatozoa. Moreover, our study clearly shows that there are no side effects in human spermatozoa caused by a sustained treatment with a pharmacological compound at 100 μM concentration and points to a specific action of A769662 on human sperm motility.

Considerable evidence shows that AMPK is deregulated in animals and humans with metabolic syndrome, type 2 diabetes, and other metabolic pathologies, as well as in cancer, and that AMPK activation can improve insulin sensitivity and metabolic health and exerts anti-cancer properties (Hardie & Alessi, 2013; Coughlan *et al.*, 2014; Pernicova & Korbonits, 2014; Rosilio *et al.*, 2014). Pharmacological agents such as metformin that are known to indirectly activate AMPK through mitochondria action are currently used to treat type 2 diabetes (Hardie & Alessi, 2013; Coughlan *et al.*, 2014; Pernicova & Korbonits, 2014; Rosilio *et al.*, 2014) and possess anti-cancer properties (Hardie & Alessi, 2013; Pernicova & Korbonits, 2014; Rosilio *et al.*, 2014). Recently, it has been also validated AMPK as a promising therapeutic target in neuron-related pathologies to treat Huntington disease (Walter *et al.*, 2016) or to treat neuropathic pain (Price & Dussor, 2013). Moreover, in a rat experimental model of inflammation, A769662 exerts anti-inflammatory effect in heart and lung tissues (Rameshrad *et al.*, 2016). In this scientific or clinical context, the present work demonstrating the specific effect of an activator of AMPK in human sperm motility might be very relevant in terms of human fertility in those patients that potentially could be treated with A769662 or other AMPK activators in the future.

Summarizing our previous (Calle-Guisado *et al.*, 2016) and present results about the role of AMPK signaling cascade in human sperm motility, we might conclude that either down fluctuation of sperm AMPK activity level below the physiological range or the treatment with AMPK activator for prolonged time plays a negative role in human sperm motility. Therefore, we propose that a specific AMPK activity (within physiological range) and kept for a proper physiological time likely plays an important and necessary regulatory role in the maintenance of the proper motility in human spermatozoa, with subsequent implications for human fertility. Moreover, the present study is important to the understanding of how human sperm motility is regulated by the AMPK activator A769662. In this regard, two facts must be considered. First, as mentioned AMPK has importantly emerged as prime target for pharmacological investigation in some cell energy-based pathologies. Secondly, this drug is currently being the most used as specific and direct AMPK activator. These two considerations and the present study point to AMPK as a key protein to be further investigated in the human sperm physiology. Future investigations, along with our current ongoing research, will determine whether physiological stimuli that clearly trigger AMPK activity in boar spermatozoa (Hurtado de Llera *et al.*, 2012, 2015; Martin-Hidalgo *et al.*, 2013) such as those leading to sperm capacitation (extracellular Ca^{2+}, bicarbonate, serum albumin) or representing sperm stresses, and probably different growth factors present in the male or female reproductive tracts, are also able to activate AMPK. Additionally, it will be relevant to elucidate which are the intracellular signaling pathways that modulate AMPK activity with the ultimate goal of elucidating the relevance of the AMPK activity assessment in human sperm for male fertility.

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AUTHORS' CONTRIBUTIONS

VC-G and AHdL performed the experiments, elaborated, and analyzed the data. LGF analyzed and discussed the data. LJG-M and MJB designed the study, analyzed and discussed the results and wrote the paper. All authors read and approved the final manuscript.

REFERENCES

Barreto-Torres G, Soto HJ, Jang S, Rodríguez-Muñoz AR, Torres-Ramos CA, Basnakian AG & Javadov S. (2015) The beneficial effects of AMP kinase activation against oxidative stress are associated with prevention of PPAR-cyclophilin D interaction in cardiomyocytes. *Am J Physiol Heart Circ Physiol* 308, H749–H758.

Calle-Guisado V, de Llera AH, Martin-Hidalgo D, Mijares J, Gil MC, Alvarez IS, Bragado MJ & Garcia-Marin LJ. (2016) AMP-activated kinase in human spermatozoa: identification, intracellular localization, and key function in the regulation of sperm motility. *Asian J Androl* 18, 1–8.

Carling D, Thornton C, Woods A & Sanders MJ. (2012) AMP-activated protein kinase: new regulation, new roles? *Biochem J* 445, 11–27.

Cool B, Zinker B, Chiou W, Kifle L, Cao N, Perham M, Dickinson R, Adler A, Gagne G, Iyengar R, Zhao G, Marsh K, Kym P, Jung P, Camp HS & Frevert E. (2006) Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. *Cell Metab* 3, 403–416.

Coughlan KA, Valentine RJ, Ruderman NB & Saha AK. (2014) AMPK activation: a therapeutic target for type 2 diabetes? *Diabetes Metab Syndr Obes* 7, 241–253.

Dang Y, Ling S, Duan J, Ma J, Ni R & Xu JW. (2015) Bavachalcone-induced manganese superoxide dismutase expression through the AMP-activated protein kinase pathway in human endothelial cells. *Pharmacology* 95, 105–110.

Evans AM, Mustard KJ, Wyatt CN, Peers C, Dipp M, Kumar P, Kinnear NP & Hardie DG. (2005) Does AMP-activated protein kinase couple inhibition of mitochondrial oxidative phosphorylation by hypoxia to calcium signaling in O2-sensing cells? *J Biol Chem* 280, 41504–41511.

Gadella BM & Harrison RA. (2000) The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behavior in the sperm plasma membrane. *Development* 127, 2407–2420.

Garrett LJ, Revell SG & Leese HJ. (2008) Adenosine triphosphate production by bovine spermatozoa and its relationship to semen fertilizing ability. *J Androl* 29, 449–458.

Goransson O, McBride A, Hawley SA, Ross FA, Shpiro N, Foretz M, Viollet B, Hardie DG & Sakamoto K. (2007) Mechanism of action of A-769662, a valuable tool for activation of AMP-activated protein kinase. *J Biol Chem* 282, 32549–32560.

Hardie DG & Alessi DR. (2013) LKB1 and AMPK and the cancer-metabolism link - ten years after. *BMC Biol* 11, 36.

Hardie DG, Schaffer BE & Brunet A. (2016) AMPK: an energy-sensing pathway with multiple inputs and outputs. *Trends Cell Biol* 26, 190–201.

Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, Frenguelli BG & Hardie DG. (2005) Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metab* 2, 9–19.

Hurtado de Llera A, Martin-Hidalgo D, Gil MC, Garcia-Marin LJ & Bragado MJ. (2012) AMP-activated kinase AMPK is expressed in boar spermatozoa and regulates motility. *PLoS ONE* 7, e38840.

Hurtado de Llera A, Martin-Hidalgo D, Rodriguez-Gil JE, Gil MC, Garcia-Marin LJ & Bragado MJ. (2013) AMP-activated kinase, AMPK, is involved in the maintenance of plasma membrane organization in boar spermatozoa. *Biochim Biophys Acta* 1828, 2143–2151.

Hurtado de Llera A, Martin-Hidalgo D, Gil MC, Garcia-Marin LJ & Bragado MJ. (2014) The calcium/CaMKKalpha/beta and the cAMP/PKA pathways are essential upstream regulators of AMPK activity in boar spermatozoa. *Biol Reprod* 90, 29.

Hurtado de Llera A, Martin-Hidalgo D, Gil MC, Garcia-Marin LJ & Bragado MJ. (2015) AMPK up-activation reduces motility and regulates other functions of boar spermatozoa. *Mol Hum Reprod* 21, 31–45.

Hurtado de Llera A, Martin-Hidalgo D, Gil MC, Garcia-Marin LJ & Bragado MJ. (2016) New insights into transduction pathways that regulate boar sperm function. *Theriogenology* 85, 12–20.

Ikematsu N, Dallas ML, Ross FA, Lewis RW, Rafferty JN, David JA, Suman R, Peers C, Hardie DG & Evans AM. (2011) Phosphorylation of the voltage-gated potassium channel Kv2.1 by AMP-activated protein kinase regulates membrane excitability. *Proc Natl Acad Sci USA* 108, 18132–18137.

Long YC & Zierath JR. (2006) AMP-activated protein kinase signaling in metabolic regulation. *J Clin Invest* 116, 1776–1783.

Martin-Hidalgo D, Hurtado de Llera A, Yeste M, Gil MC, Bragado MJ & Garcia-Marin LJ. (2013) Adenosine monophosphate-activated kinase, AMPK, is involved in the maintenance of the quality of extended boar semen during long-term storage. *Theriogenology* 80, 285–294.

Moreno D, Knecht E, Viollet B & Sanz P. (2008) A769662, a novel activator of AMP-activated protein kinase, inhibits non-proteolytic components of the 26S proteasome by an AMPK-independent mechanism. *FEBS Lett* 582, 2650–2654.

Nguyen TM, Alves S, Grasseau I, Metayer-Coustard S, Praud C, Froment P & Blesbois E. (2014) Central role of 5'-AMP-activated protein kinase in chicken sperm functions. *Biol Reprod* 91, 121.

Pernicova I & Korbonits M. (2014) Metformin–mode of action and clinical implications for diabetes and cancer. *Nat Rev Endocrinol* 10, 143–156.

Price TJ & Dussor G. (2013) AMPK: an emerging target for modification of injury-induced pain plasticity. *Neurosci Lett* 557, 9–18.

Rajamohan F, Reyes AR, Frisbie RK, Hoth LR, Sahasrabudhe P, Magyar R, Landro JA, Withka JM, Caspers NL, Calabrese MF, Ward J & Kurumbail RG. (2016) Probing the enzyme kinetics, allosteric modulation and activation of alpha1- and alpha2-subunit-containing AMP-activated protein kinase (AMPK) heterotrimeric complexes by pharmacological and physiological activators. *Biochem J* 473, 581–592.

Rameshrad M, Soraya H, Maleki-Dizaji N, Vaez H & Garjani A. (2016) A-769662, a direct AMPK activator, attenuates lipopolysaccharide-induced acute heart and lung inflammation in rats. *Mol Med Rep* 13, 2843–2849.

Rosilio C, Ben-Sahra I, Bost F & Peyron JF. (2014) Metformin: a metabolic disruptor and anti-diabetic drug to target human leukemia. *Cancer Lett* 346, 188–196.

Sanders MJ, Ali ZS, Hegarty BD, Heath R, Snowden MA & Carling D. (2007) Defining the mechanism of activation of AMP-activated protein kinase by the small molecule A-769662, a member of the thienopyridone family. *J Biol Chem* 282, 32539–32548.

Scott JW, Hawley SA, Green KA, Anis M, Stewart G, Scullion GA, Norman DG & Hardie DG. (2004) CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. *J Clin Invest* 113, 274–284.

Scott JW, van Denderen BJ, Jorgensen SB, Honeyman JE, Steinberg GR, Oakhill JS, Iseli TJ, Koay A, Gooley PR, Stapleton D & Kemp BE. (2008) Thienopyridone drugs are selective activators of AMP-activated protein kinase beta1-containing complexes. *Chem Biol* 15, 1220–1230.

Suter M, Riek U, Tuerk R, Schlattner U, Wallimann T & Neumann D. (2006) Dissecting the role of 5'-AMP for allosteric stimulation, activation, and deactivation of AMP-activated protein kinase. *J Biol Chem* 281, 32207–32216.

Tartarin P, Guibert E, Toure A, Ouiste C, Leclerc J, Sanz N, Briere S, Dacheux JL, Delaleu B, McNeilly JR, McNeilly AS, Brillard JP, Dupont J, Foretz M, Viollet B & Froment P. (2012) Inactivation of AMPKalpha1 induces asthenozoospermia and alters spermatozoa morphology. *Endocrinology* 153, 3468–3481.

Walter C, Clemens LE, Muller AJ, Fallier-Becker P, Proikas-Cezanne T, Riess O, Metzger S & Nguyen HP. (2016) Activation of AMPK-induced autophagy ameliorates Huntington disease pathology in vitro. *Neuropharmacology* 108, 24–38.

Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, Schlattner U, Wallimann T, Carlson M & Carling D. (2003) LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol* 13, 2004–2008.

Xie M, Zhang D, Dyck JR, Li Y, Zhang H, Morishima M, Mann DL, Taffet GE, Baldini A, Khoury DS & Schneider MD. (2006) A pivotal role for endogenous TGF-beta-activated kinase-1 in the LKB1/AMP-activated protein kinase energy-sensor pathway. *Proc Natl Acad Sci USA* 103, 17378–17383.

