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ARTICLE

The sirtuin 1 activator YK 3-237 stimulates capacitation-related events in human spermatozoa

BIOGRAPHY

David Martín-Hidalgo is a biology reproductive researcher with a veterinary background. He is interested in improving the outcome of assisted reproductive technology in mammals, mainly humans and domestic animals. His research and expertise have been focused on the study of sperm metabolism and intracellular pathways as a tool to overcome male infertility.

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KEY MESSAGE

This is the first study to show the role of sirtuins in the human sperm capacitation pathway. Human spermatozoa SIRT1 activated by YK 3-237 enhances capacitation-related events by bringing forward and increasing the levels of protein tyrosine phosphorylation and increasing the percentage of spermatozoa acrosome reacted after calcium ionophore challenge.

ABSTRACT

Research question: Does sirtuin-1 (SIRT1) have a role in the human spermatozoa capacitation process?

Design: Human spermatozoa were incubated for 6 h in a capacitating medium in presence or absence of the specific SIRT1 activator, YK 3-237. Several sperm parameters were determined by flow cytometry: viability, acrosome reaction and mitochondria membrane status. Sperm motility was determined objectively by computer-assisted semen analysis. Sperm capacitation status was evaluated by the extent of protein tyrosine phosphorylation and by the percentage of spermatozoa with the acrosome reacted by a calcium ionophore challenge.

Results: SIRT1 was detected in the connecting piece of human spermatozoa where a lysine acetylation pattern was mainly found along the sperm tail. SIRT1 activation accelerates the occurrence of a phenotype associated with human sperm capacitation, with no differences seen in the lysine acetylation pattern. After 1 h of co-incubation of YK 3-237 with human spermatozoa, tyrosine phosphorylation levels were comparable to control levels after 6 h of incubation in capacitating conditions. In addition, the activator improved sperm responsiveness to a Ca^{2+} ionophore (A23187) challenge determined by an increase in acrosome-reacted spermatozoa ($P = 0.025$). Importantly, sperm viability and mitochondrial activity-related parameters assessed by flow cytometry were not affected by YK 3-237.

Conclusion: YK 3-237 induces capacitation-related events in human spermatozoa such an increase of phosphorylation levels and acrosome-reacted spermatozoa after the ionophore challenge. Together, these results show that YK 3-237 affects human spermatozoa capacitation-related events by a mechanism independent of protein lysine acetylation but dependent on bicarbonate and calcium.

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1472-6483/
Declaration: The authors report no financial or commercial conflicts of interest.

KEYWORDS

Acrosome reaction
Capacitation
Human spermatozoa
Protein tyrosine phosphorylation
Sirtuin-1

INTRODUCTION

As society grows, there is constant growth in the number of couples seeking aid and reproductive counsel, with 50% of fertility issues having their origin in men (*Agarwal et al., 2015*). In this context, there is an urgent need for the development of methods that improve male fertility, to enhance the current low yield of treatment with assisted reproductive technologies (ART). Current ART protocols are based on the findings described in 1951 by Austin and Chang, who independently resolved the cornerstone for IVF procedures. Both researchers observed that spermatozoa deposited into the oviduct were not able to fertilize the oocyte immediately and needed a period of time in the female tract in order to acquire fertility potential, a process named sperm capacitation (*Austin, 1951*; *Chang, 1951*). Sperm capacitation is characterized by the increase in post-translational modifications (mostly protein tyrosine phosphorylation) of a wide range of proteins and by their ability to undergo the acrosome reaction (*Arcelay et al., 2008*; *Blackmore, 1993*; *Yanagimachi, 1970*).

Despite the increased rates of successful ART, there is still ample scope for improvement. Specifically, human pregnancy rates are only 28% and 25% for IVF and intracytoplasmic sperm injection, respectively, with the delivery rate of intrauterine insemination being 8.9% (*Wyns et al., 2020*). One of the possible origins of this low efficiency might be associated with sperm capacitation pathway deregulation (*Schinfeld et al., 2018*; *Sharara et al., 2020*). This hypothesis is supported by the fact that although men whose ejaculates meet the requirements of the World Health Organization (WHO) to be considered as normozoospermic, they can still be infertile (*Van Geloven et al., 2013*). Hence, the challenge for optimizing current knowledge about human sperm molecular mechanisms triggered in a capacitation medium represents an imperative need to face male infertility associated with a failure in sperm capacitation.

Interestingly, there are differences between species in the incubation time necessary to achieve maximum levels of protein tyrosine phosphorylation: 2 h in the mouse (*Goodson et al., 2012*), 6–18 h in humans (*Battistone et al., 2014*; Calle-Guisado *et al.*, 2018; *Matamoros-Volante et al., 2018*) and 4 h in the pig (*Bravo et al., 2005*). The wide variety of human sperm capacitation behaviours is shown by this wide range of times. Some cohorts of male ejaculates capacitate very early (early capacitation status), while others capacitate too late (late capacitation status) in comparison with the average for the male population, highlighting the issues associated with deregulation of sperm capacitation in men (*Ostermeier et al., 2018*). Thus, men from both groups might present difficulties when using ART to overcome fertility issues because the timing of the ART procedures are standardized and fixed (*Ostermeier et al., 2018*). Due to this protocol standardization, there is often a high failure rate for patients seeking ART with samples that present non-standard behaviours. In fact, the classic spermiogram is not always sufficient to predict the fertilizing capacity of an ejaculate because many infertile men have a normal spermiogram (*Van Geloven et al., 2013*). There is a growing consensus that male infertility cannot be assessed by conventional analysis of sperm quality alone, but also requires the assessment of the sperm's ability to efficiently undergo key molecular events, such as capacitation.

Sperm capacitation signalling pathways are highly orchestrated, with the aim of increasing the chances of spermatozoa fertilizing an oocyte. Classic studies on sperm capacitation have been focused on protein phosphorylation/dephosphorylation events (*Porambo et al., 2012*). Only recently has lysine acetylation been shown to also have a key role in capacitation signalling in human (*Sun et al., 2014*; *Yu et al., 2015*), mouse (*Ritagliati et al., 2018*) and pig sperm conservation (*Chen et al., 2021*). These findings underscore the need for a deeper study of this signalling pathway to unravel the role in sperm physiology. Regulation of acetylation/deacetylation is driven by sirtuins (SIRT), a family of deacetylase proteins highly conserved in the animal kingdom (*Michan and Sinclair, 2007*). The functions of sirtuins in reproductive processes were unveiled when both male and female SIRT1-null mice were found to be sterile (*McBurney et al., 2003*). From the point of view of male reproductive health, sirtuins have been implicated in the control of spermiogenesis (*Bell et al., 2014*; *Coussens et al., 2008*; *Kolthur-Seetharam et al., 2009*), male germ cell differentiation, testis development (*Bell et al., 2014*) and, more recently, in sperm acrosome biogenesis (*Liu et al., 2017*). In addition, sirtuins play key roles in ageing (*Chen et al., 2020*; *Lee et al., 2019*) and diseases (*Deng et al., 2019*; *Granchi and Minutolo, 2018*; *Ponnusamy et al., 2015*; *Yi et al., 2013*; *Zhang et al., 2020*) making them an attractive target to enhance sperm features. Nevertheless, their effects on mature spermatozoa remain unexplored.

This study aimed to investigate the role of the SIRT1 activator, YK 3-237, on essential functional sperm parameters related to the capacitation process of human spermatozoa. This is the first report to focus on a possible role for a SIRT1 activator on human sperm capacitation-like functional processes.

MATERIALS AND METHODS

Chemicals

Sperm Wash Gradient Set (45% and 90%) was purchased from Merck (Darmstadt, Germany). Propidium iodide, SYBR-14 and MitoSOX™ Red probes were purchased from Thermo Fisher Scientific (Waltham, MA, USA); bovine serum albumin (BSA, fatty acid-free), PNA-FITC and Anti-Phosphotyrosine Antibody (clone 4G10®) from Sigma-Aldrich (St Louis, MO, USA); JC-1 probe from Thermo Fisher Scientific; ISOTON II Diluent from Beckman Coulter Inc. (Brea, CA, USA); DC™, 2x Laemmli Sample Buffer and Clarity™ Western ECL Substrate from Bio-Rad Laboratories Inc. (Hercules, CA, USA). Restore™ PLUS Western Blot Stripping Buffer were from Thermo Fisher Scientific (Rockford, USA). PKI (14-22) amide (myristoylated) was obtained from Enzo Life Sciences, Inc. (New York, USA); LRE1 and YK 3-237 were purchased from Sigma-Aldrich; Alexa Fluor® 488 goat anti-mouse IgG was from Life Technologies Ltd (Grand Island, NY, USA); 4,6-diamidino-2-phenylindole hydrochloride (DAPI, 125 µg/l) from Invitrogen Molecular Probes (Grand Island, NY, USA); SIRT1 (#ab32441) antibody was purchased from Abcam; Acetylated-Lysine Antibody (#9441) was obtained from Cell Signaling (Danvers, MA, USA).

Human samples and ethical issues

Human seminal samples were obtained from 86 normozoospermic men, with

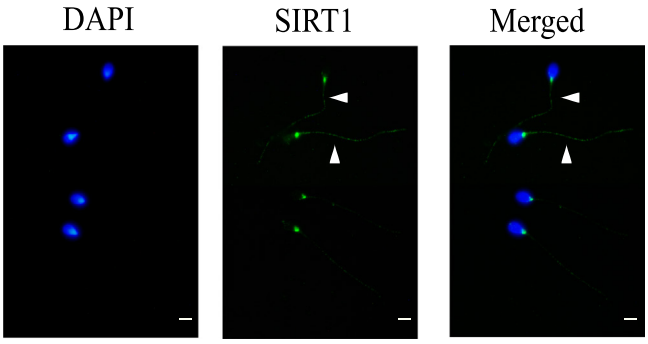

FIGURE 1 Immunofluorescence staining of SIRT1 in human mature spermatozoa – SIRT1 protein is localized mainly in the connecting piece of mature human spermatozoa. On the left panel in blue is depicted the nuclei of four different human spermatozoa stained with DAPI. The middle panel shows the localization of SIRT1 (green). Note that all spermatozoa show a stronger localization in the connecting piece, although some of them show SIRT1 localized along the sperm tail (white triangles); other spermatozoa do not show this staining. The right panel shows the merging of both fluorescent images; the DAPI intensity is diminished in the merged images. Scale bar in white: 5 µm. DAPI = 4,6-diamidino-2-phenylindole; SIRT1 = sirtuin-1.

no known disease, from couples seeking infertility treatment at the Centre for Reproductive Genetics Alberto Barros (Porto, Portugal). Patient selection, clinical study and diagnostic semen analysis were performed at the clinic. All procedures were conducted in accordance with the ethical guidelines for human samples research with informed and written consent obtained from all individual donors included in the study. This study did not involve direct experiments on humans or animals, as only donated samples from surplus fresh ejaculated semen samples collected for diagnostic purposes were used. The approval of the Ethics Committee and the Declaration of Helsinki on human experimentation, revised in Tokyo 2004, does not apply to this work. The procedures of the Centre for Reproductive Genetics Alberto Barros are covered by the provisions of the National Medically Assisted Procreation Act (2017) and overseen by the National Council for Medically Assisted Procreation (CNPMA-2018). According to these rules and guidelines, the use of clinical databases and patient biological material for diagnosis and research may be used without further ethical approval, under strict individual anonymity, and after patient written informed consent. Regarding the use of semen samples for laboratory experimentation at ICBAS-UP, the Ethics Committee authorization number is 2021/CE/P02 (P342/2021/CETI), approved on 26 February 2021.

Human sperm culture medium

For sperm culture, Biggers–Whitten–Whittingham (BWW) medium was used, as described by *Biggers et al. (1971)* with slight modifications. BWW-washed (BWW-W, in mmol/l): 94.5 NaCl, 4.8 KCl, 1.7 $CaCl_2.2H_2O$, 1.17 KH_2PO_4, 1.22 $MgSO_4.7H_2O$, 20 HEPES. Non-capacitation medium contains all the components of BWW-W plus 5.56 mmol/l glucose and gentamycin 10 µg/ml. Capacitating medium contains all the components of non-capacitation medium plus 25 mmol/l of HCO_3^- and 26 mg/ml fatty acid-free BSA. All the media were adjusted to a final pH of 7.2–7.4.

Human sperm preparation

Semen samples were collected by masturbation into sterile cups after 2–4 days of sexual abstinence. Samples were left to settle for up to 2 h at 37°C to allow liquefaction prior to processing, as described below. Only ejaculates whose semen parameters (total fluid volume, sperm concentration, motility and morphology) met the normality criteria established by the *WHO (2010)* were processed. Sperm viability (FIGURE 1) was determined by eosin–nigrosin staining following the instructions of the WHO manual (2010). Ejaculates were submitted to discontinuous gradient centrifugation (Sperm Wash Gradient Set (45% and 90%)) for 20 min at 300*g* at room temperature. The bottom gradient layer (purified populations of highly motile spermatozoa) was recovered and washed twice (500*g* for 5 min at room temperature) with modified BWW-W medium containing no substrates for energy metabolism. The pellet was then resuspended in 1 ml of BWW-W medium and sperm concentration was determined using a Neubauer counting chamber under an optical microscope (100 × magnification). Different ejaculates were pooled to perform western blot experiments; the remaining assays (flow cytometry and motility) were performed on each single ejaculate. Finally, according to the experimental design, spermatozoa were diluted in 1 ml of capacitating (BWW medium containing 26 mg/ml of BSA and 25 mmol/l of bicarbonate) or non-capacitating medium (BWW) at a final concentration of 20×10^6/ml for 1 or 6 h at 37°C in the presence or absence of activators and/or inhibitors. When needed, inhibitors were pre-incubated for 15 min at 37°C in non-capacitating conditions before the addition of equal parts (1:1) of capacitating medium (2 ×).

Human sperm motility analysis

At different time points (1 and 6 h), 6 µl of sperm sample (20×10^6/ml) incubated in the presence or absence of the SIRT1 activator (10 µmol/l YK 3-237) at 37°C was placed in a pre-warmed Spermtrack counting chamber and the following motility parameters were evaluated using a computer-assisted semen analysis (CASA) system and the ISAS® software (PROISER, Paterna, Valencia, Spain): percentage of motile and progressive spermatozoa, curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), amplitude of lateral sperm head movement (ALH), linearity coefficient (LIN), straightness coefficient (STR), wobble movement coefficient (WOB) and beat cross frequency (BCF). The settings of the CASA system were as follows: frame rate, 60 Hz; frame acquired, 25; straightness threshold, 80%. The total number of spermatozoa evaluated in each semen sample was at least 300.

Human sperm acrosome responsiveness to A23187 calcium ionophore challenge

Human sperm acrosome reaction evaluation after an ionophore challenge was performed following WHO guidelines (*World Health Organization, 2010*) with slight modifications. Briefly, after 3 h of incubation in capacitating medium at 37°C in the presence or absence of YK 3-237 (10 µmol/l), 100 µl human spermatozoa were diluted with 400 µl BWW-W medium and incubated in the presence of calcium ionophore A23187 at a final concentration of 10 µmol/l for 15 min. Afterwards, spermatozoa were washed with 1 ml capacitating medium (5 min at 1800*g*), to remove A23187 from

the medium and stop progression of the acrosome reaction. Acrosome reaction was immediately determined by flow cytometry as described in the section below.

Analysis of human sperm functional parameters by flow cytometry

In brief, to analyse each parameter, 200,000 spermatozoa for each treatment (control or YK 3-237) were incubated in BWW-W medium (in the dark) with: 20 nmol/l of SYBR-14 probe and 9.6 µmol/l of propidium iodide for 20 min at room temperature, to measure sperm viability; 1.25 µg/ml of PNA-FITC and 24 µmol/l of propidium iodide for 5 min at room temperature, to measure acrosome membrane integrity; 2 µmol/l of MitoSOX™ for 15 min at 37°C, to measure mitochondrial anion superoxide production; 1.5 µmol/l of JC-1 for 1 h at 37°C, to evaluate mitochondrial membrane potential.

For the analysis, a flow cytometer was used (ACEA NovoCyte™, ACEA Biosciences, Inc., San Diego, CA, USA) containing the ACEA NOVOEXPRESS™ software. The fluorescence values of SYBR-14 and PNA-FITC were collected in the laser-excited fluorescence channel (BL1) using a 530 ± 30-nm band-pass filter, whereas MitoSOX fluorescence was collected in the BL2 channel using a 572 ± 28-nm band-pass filter and propidium iodide fluorescence was collected in the BL4 channel using a 675 ± 30-nm band-pass filter. The results were expressed as the average of the percentage of labelled spermatozoa for each parameter analysed. Sperm viability was considered as the percentage of SYBR-14$^+$ and PI$^-$ labelled cells. Mitochondrial anion superoxide production was considered as the percentage of MitoSOX$^+$. Acrosome membrane integrity was evaluated as the percentage of PNA-FITC$^+$ and PI$^-$ labelled spermatozoa. The fluorescence values of JC-1 were collected in the channels BL1 and BL2 using band-pass filters as described above. The percentage of orange-stained cells was recorded and considered the population of spermatozoa with a high mitochondrial membrane potential. The results are expressed as the average of the percentage of orange-stained spermatozoa.

Protein detection by immunofluorescence

A total of 8 × 10^5 spermatozoa were mounted on a slide and fixed with 4% paraformaldehyde for 30 min, washed with phosphate-buffered saline (PBS), permeabilized with Triton X-100 (0.25%, v/v) for 10 min, washed again and blocked with BSA (3%, w/v) for 30 min. Incubations with anti-phosphotyrosine antibody (1:250), anti-SIRT1 antibody (1:200) or with anti-acetyl-lysine antibody (1:200) were carried out overnight at 4°C. After washing the slides with PBS, samples were incubated with Alexa Fluor 488 goat anti-rabbit IgG (1:250) or anti-mouse IgG (1:250) for 120 min at room temperature. As negative controls, samples incubated with the secondary antibody and without the primary antibody were run in parallel (Supplementary Figure 1). Coverslips were washed and mounted on slides containing 5 µl DAPI with an antifading mounting solution. The coverslips were finally sealed, and the slides stored until microscope observation. Fluorescence images were obtained using a Nikon Eclipse 50i fluorescence microscope (Nikon, Shinagawa, Tokyo, Japan). All pictures were captured with the same exposure time and gain.

Western blot analysis

Briefly, samples from human spermatozoa were washed in PBS for 3 min at 5000*g* and then lysated in 2 × Laemmli Sample Buffer for 30 min at 4°C. The homogenates were subjected to centrifugation at 10,000*g* for 10 min at 4°C and the supernatant, containing the solubilized sperm proteins, was used for protein analysis. Protein concentration was determined using the DC™ Protein Assay (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. Sperm proteins (15 µg) were resolved using 10% SDS-PAGE (120V for 90 min) and electro-transferred by semi-dry method (2.5A for 8 min) to PVDF membranes. Immunoblotting was conducted with anti-pY (clone 4G10), anti-SIRT1 or anti-acetyl-lysine. In brief, PVDF membranes were blocked with 5% BSA in TBS (Tris-HCl 20 mmol/l, NaCl 150 mmol/l, pH 7.5) containing 0.1% Tween 20 (T-TBS). Anti-pY antibody was used at a final dilution of 1:2500, whereas anti-SIRT1 and anti-acetyl-lysine were used at dilutions of 1:1000. Secondary antibodies (anti-mouse for phosphotyrosine or anti-rabbit for SIRT and acetyl-lysine) were diluted in T-TBS (1:5000). Blots were visualized with Clarity™ Western ECL Substrate (Bio-Rad) and measured using a ChemiDoc XR system (Bio-Rad). Densities from each band were quantified using Image Lab Software (Bio-Rad). Western blot analysis regions of interest used for quantification are indicated by a vertical bar on the left of the respective western blot. Images shown are representative of experiments repeated at least three times ($n \geq 3$) using a combination of ejaculates from three different men.

Statistical analysis

Data were assessed for normality using a Shapiro–Wilk test and analysed for equal variances. Differences between treatments were analysed by one-way analysis of variance (ANOVA). When more than one condition was analysed (time and treatment), a two-way ANOVA was performed. When significance was found, post-hoc comparisons between treatments were made through Bonferroni's test. Results were considered significant when *P*-values were <0.05. Results are expressed as the mean of at least four independent experiments plus SEM and were calculated for descriptive statistics. Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, CA).

RESULTS

SIRT1 is localized in the human sperm neck and its activation promotes protein tyrosine phosphorylation through a mechanism independent of lysine acetylation

SIRT1 was detected by immunofluorescence in the connecting piece of the neck region of human spermatozoa, and a few spermatozoa also showed a faint signal along the tail (FIGURE 1). Once the presence of SIRT1 was identified in human spermatozoa, viability in the presence of the SIRT1 activator, YK 3-237, was studied. Thus, spermatozoa were incubated in capacitating conditions for 6 h with different concentrations of YK 3-237 (from 0 to 60 µmol/l). After 6 h of incubation, no effect on sperm viability was observed regardless of the concentration used (FIGURE 2A). Interestingly, YK 3-237 induced an increase in phosphotyrosine concentrations compared to control samples, with higher values observed after exposure to 5 (P = 0.284), 10 (P = 0.037) and 30 µmol/l (P = 0.0295) YK 3-237 (3.2, 3.7 and 3.7 times higher, respectively) (FIGURE 2B). Therefore, it was

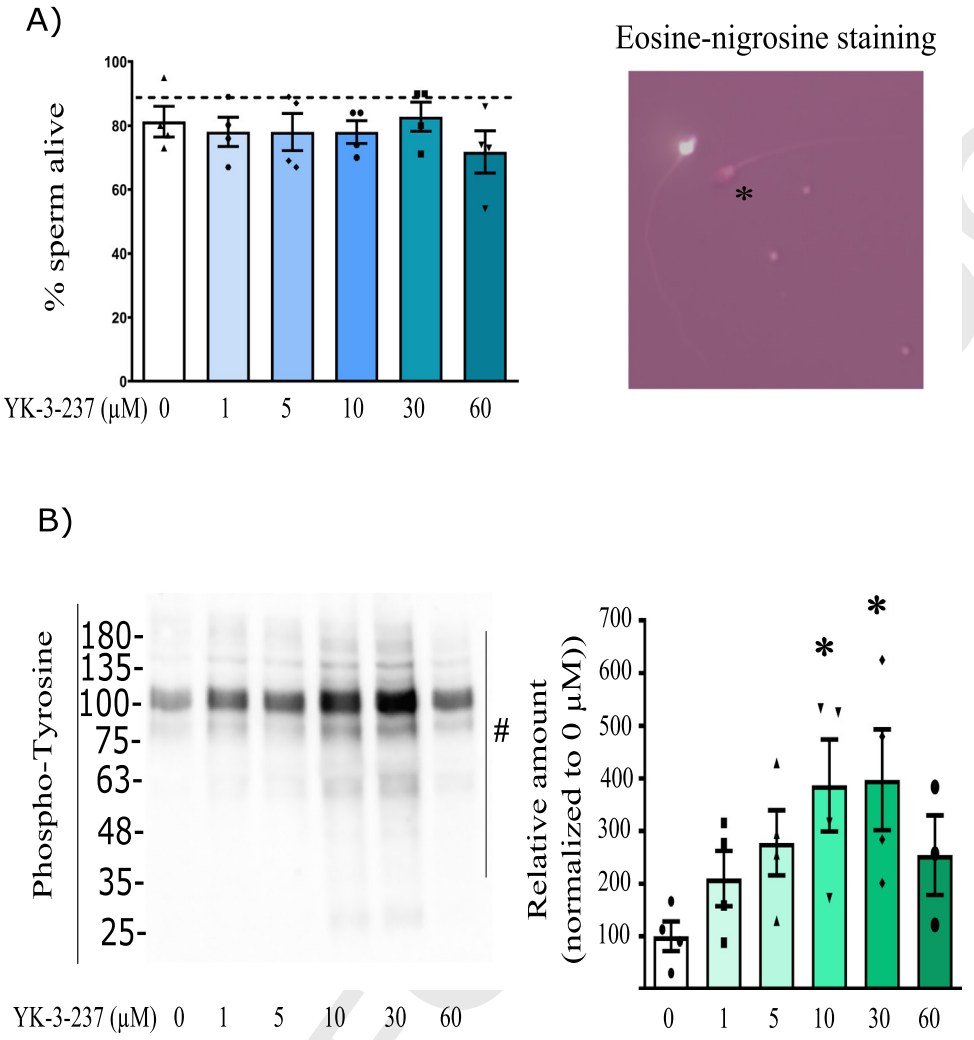

FIGURE 2 YK 3-237 increases phosphotyrosine protein concentrations in human spermatozoa without affecting sperm viability. Human spermatozoa were incubated for 6 h at 37°C under capacitating medium (BWW-modified medium supplemented with HCO_3^- 25 mmol/l and BSA 26 mg/ml) in the presence of the indicated concentrations of YK 3-237. (A) Sperm viability of human spermatozoa incubated at different concentrations of YK 3-237 determined by eosin–nigrosin stain (n = 4). The dotted line shows initial sperm viability before initiating sperm incubation. Right panel: representative human spermatozoa stained with eosin–nigrosin stain. Spermatozoa with white background is an example of a live spermatozoon whereas those with pink backgrounds (*) are dead spermatozoa. (B) Left panel: a representative western blot using anti-phosphotyrosine antibody in human spermatozoa incubated with different concentrations of YK 3-237 in capacitating medium for 6 h. Right panel: western blots were analysed using Image Lab (n = 4). For comparison between blots, pixels for each lane contained in the region marked by # (proteins in a 35–135 kDa range) were quantified and normalized using the capacitating medium lane (HCO_3^- 25 mmol/l, BSA 26 mg/ml in the absence of YK 3-237) as reference (100%). Bars represent the average ± SEM. Data were analysed statistically by one-way analysis of variance. When statistical differences versus control conditions (HCO_3^- 25 mmol/l, BSA 26 mg/ml in the absence of YK 3-237) were found, the P-values are reported in the graphic. µM = µmol/l. BSA = bovine serum albumin; BWW = Biggers–Whitten–Whittingham.

decided to use 10 µmol/l YK 3-237 in the subsequent experiments, as it was the lowest concentration tested for the SIRT1 activator that prompted a large increase in phosphotyrosine concentrations in human spermatozoa.

Because sirtuin family members function as lysine deacetylases, the lysine acetylation pattern in human spermatozoa in the presence or absence of YK 3-237 (10 µmol/l) was evaluated (by western blotting and immunofluorescence).

Immunofluorescence experiments revealed that lysine acetylation is found along the sperm tail, with a reduced number of spermatozoa also showing fluorescence in the equatorial segment of the head (FIGURE 3A). An intense lysine acetylation pattern was observed in the western blotting experiments, at a molecular weight close to 50 kDa. No differences were found in the acetylation of lysine residues on human spermatozoa incubated in capacitating conditions in the presence or absence of YK 3-237 (FIGURE 3A and 3B).

YK 3-237-mediated phosphotyrosine enhancement on human spermatozoa is dependent on bicarbonate and calcium

Because of the clear effect of YK 3-237 on tyrosine phosphorylation, it was decided to evaluate the role of this activator under different capacitating stimuli in human spermatozoa. Bicarbonate, calcium and BSA are the components that trigger the signalling cascade necessary for sperm capacitation, which will eventually lead to increased protein tyrosine

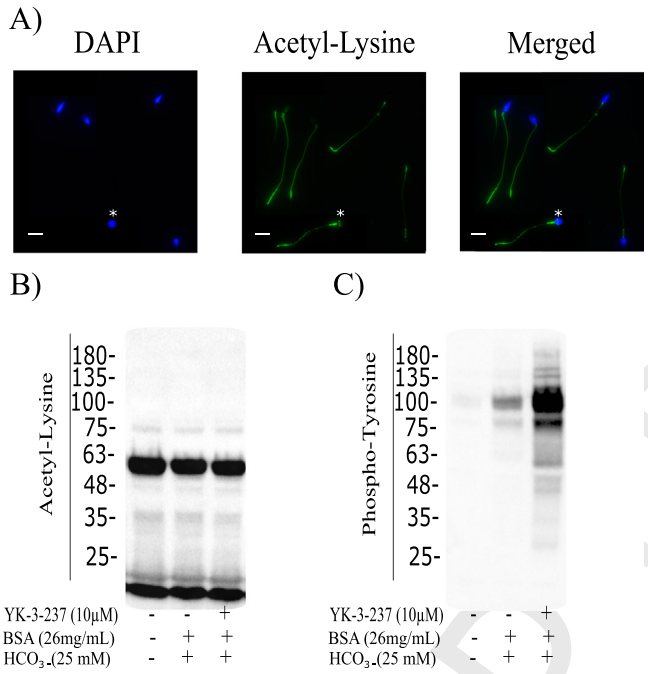

FIGURE 3 YK 3-237 induces protein tyrosine phosphorylation that is independent of protein lysine acetylation. Human spermatozoa were incubated for 6 h at 37°C under different media, which support or do not support human sperm capacitation (presence or absence of HCO_3^- 25 mmol/l and BSA 26 mg/ml) with or without YK 3-237 (10 µmol/l). (A) Immunofluorescence of acetylated proteins in human mature spermatozoa. On the left panel is depicted the nuclei of different human spermatozoa stained with DAPI (blue). The middle panel shows the localization of acetylated proteins (green), mainly along the tail of the spermatozoa. Some spermatozoa show acetylated proteins on the equatorial segment of the head (white asterisk). The right panel shows the merging of both fluorescence images. Scale bar in white: 5 µmol/l. (B) A representative western blot using anti-acetyl-lysine antibody in human spermatozoa incubated under indicated conditions. (C) A representative western blot using anti-phosphotyrosine antibody in human spermatozoa incubated under indicated conditions. µM = µmol/l. BSA = bovine serum albumin; DAPI = 4,6-diamidino-2-phenylindole.

phosphorylation (reviewed by *Gervasi and Visconti, 2016*). To evaluate their individual roles on phosphotyrosine induced by the activator YK 3-237, human spermatozoa were incubated in the absence or presence of BSA (26 mg/ml), bicarbonate (25 mmol/l) and YK 3-237 (10 µmol/l). While co-incubation of the activator with BSA alone did not affect phosphotyrosine concentrations (FIGURE 4A), the presence of bicarbonate in the medium tripled the phosphotyrosine concentrations found in the control sample ($P = 0.016$). Furthermore, the effects on phosphotyrosine concentrations were augmented (four-fold increase) when spermatozoa were incubated with both bicarbonate and BSA ($P < 0.001$) in the presence of YK 3-237 (FIGURE 4A and 4B).

Due to the YK 3-237 enhancing effects on human sperm phosphotyrosine concentrations after 6 h in a complete capacitating medium, sperm phosphotyrosine concentrations at different time points were evaluated to determine a threshold. Although YK 3-237 rapidly induced an increase in phosphotyrosine concentrations after just 15 min, this increase started to be statistically significant ($P = 0.023$–0.0001) from 45 min onwards until the end (FIGURE 4C and 4D). It is worth highlighting that, after 1 h of incubation with YK 3-237, phosphotyrosine concentrations doubled when compared with those observed in human spermatozoa incubated for 6 h in capacitating control conditions (FIGURE 4C and 4D). These results were further confirmed by immunofluorescence, as depicted in FIGURE 5. Clearly, an increase ($P = 0.012$) in the number of phosphotyrosine-positive spermatozoa is observed in the presence of YK 3-237 (10 µmol/l), compared to spermatozoa incubated for 6 h without YK 3-237.

Importantly, calcium is needed for sAC activation and has a biphasic role in spermatozoa of several species, such as mouse (*Navarrete et al., 2015*), horse (*González-Fernández et al., 2012*) and human (*Baker et al., 2004*; *Marín-Briggiler et al., 2003*). In the current study conditions, when human spermatozoa were incubated without added calcium, nominal zero calcium (still containing micromolar concentrations of calcium (*Marin-Briggiler et al., 2005*), or with absolutely no calcium chelated by EGTA, increasing phosphotyrosine concentrations were 3.2 ($P = 0.031$) and 4.5 ($P = 0.001$) times higher, respectively, in comparison with the control (1.7 mmol/l Ca^{2+}) (FIGURE 6). Spermatozoa incubated in nominal zero calcium in the presence of YK 3-237 showed increased phosphotyrosine concentrations, while those spermatozoa incubated in EGTA plus YK 3-237 did not exhibit any significant increase in phosphotyrosine concentrations, compared with those in the absence of YK 3-237 (FIGURE 6).

YK 3-237 acts downstream of sAC/PKA in the pathway leading to phosphotyrosine in human spermatozoa

Due to the effects of YK 3-237 on phosphotyrosine concentrations (one of the hallmarks of sAC/PKA pathway activation) and in order to gain knowledge about the impact of YK 3-237 on the sAC/PKA pathway,

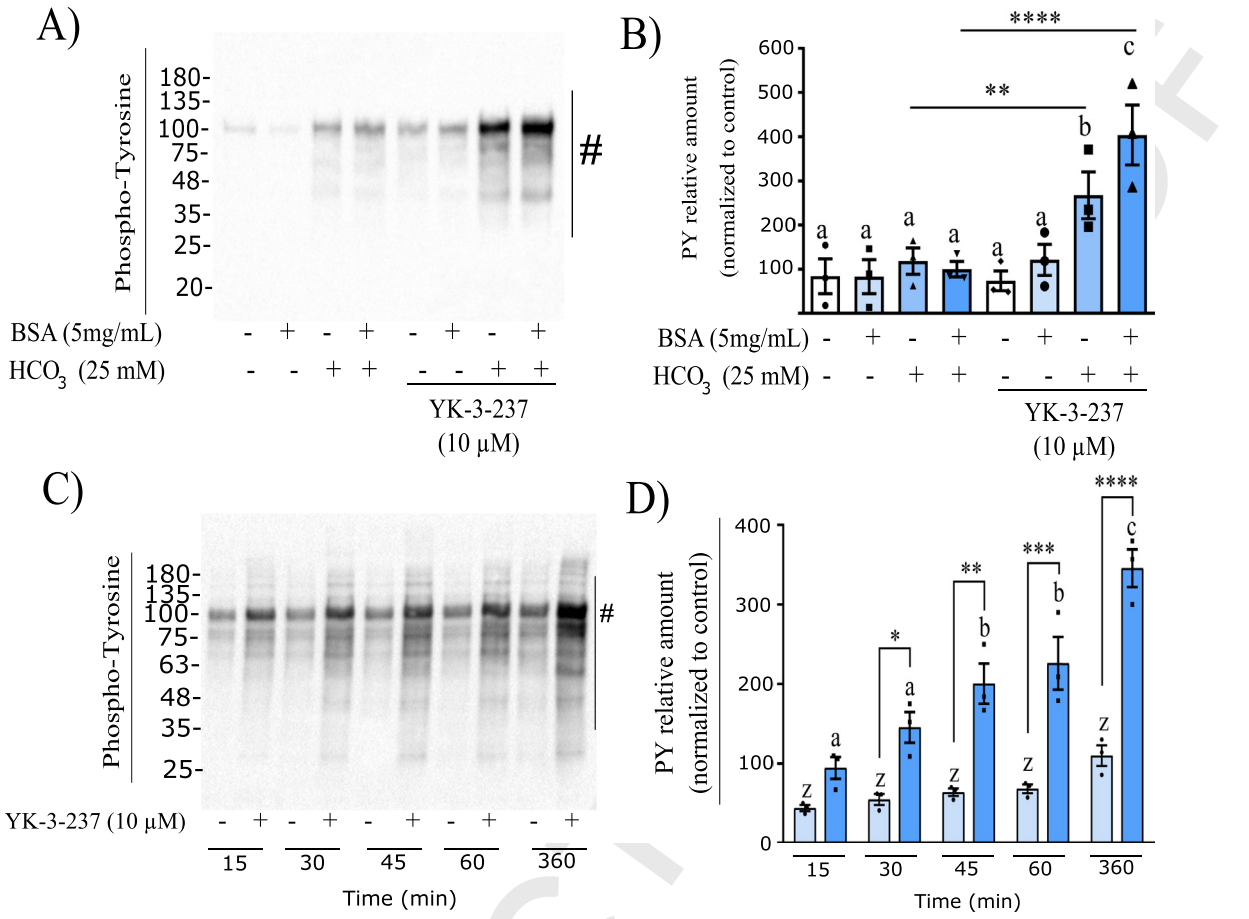

FIGURE 4 YK 3-237-induced tyrosine phosphorylation is dependent on bicarbonate. Human spermatozoa were incubated for 6 h at 37°C under indicated conditions that support or do not support human sperm capacitation. (A) A representative western blot using anti-phosphotyrosine antibody of human spermatozoa incubated under indicated conditions (presence or absence of HCO_3^- 25 mmol/l and BSA 26 mg/ml) with or without YK 3-237 (10 µmol/l) (n = 3) is shown. (B) Western blots were analysed using Image Lab. For comparison between blots, pixels for each lane contained in the region marked in (A) by # (proteins in a 35–135 kDa range) were quantified and normalized using the control (HCO_3^- 25 mmol/l, BSA 26 mg/ml and YK 3-237 0 µmol/l) lane as reference (100%). Bars represent the average ± SEM. Data were analysed statistically by one-way analysis of variance. Bonferroni post-hoc test was used to analyse differences between the presence or absence of YK 3-237 (10 µmol/l). When differences were found the P-values are reported in the graphic. Different superscripts ^{a,b,c} show differences between treatments. (C) A representative western blot using anti-phosphotyrosine antibody of human spermatozoa incubated during indicated time points and conditions (HCO_3^- 25 mmol/l and BSA 26 mg/ml) in presence or absence of YK 3-237. (D) Western blots were analysed using Image Lab. Light blue bars represent control conditions; dark blue bars represent YK 3-237 treatment. Bars represent the average ± SEM (n = 3). For comparison between blots, pixels for each lane contained in the region marked by # in (C) (proteins in a 35–135 kDa range) were quantified and normalized using control conditions (HCO_3^- 25 mmol/l, BSA 26 mg/ml and YK 3-237 0 µmol/l) at 6 h lane as reference (100%). Data were analysed statistically by one-way analysis of variance. Bonferroni post-hoc test was used to analyse differences between the presence or absence of YK 3-237 (10 µmol/l); when differences between control and YK 3-237 conditions within the same time points was found, the P-value is reported in the graphic. Different superscripts ^{a,b,c} show differences between YK 3-237 treatments with time. Different superscripts ^{z,y} show differences between control samples with time. µM = µmol/l; mM = mmol/l. BSA = bovine serum albumin; PY = phosphotyrosine.

human spermatozoa were incubated in the presence and absence of sAC or PKA inhibitors: LRE1 (30 µmol/l) and PKI (30 µmol/l), respectively. As shown in FIGURE 7, the effect of those inhibitors on the phosphotyrosine signal appears partially reverted by the presence of YK 3-237. However, due to high variability, some of the changes were not statistically significant when using a Bonferroni correction. For instance, phosphotyrosine was reduced (normalized value to line 1 (control) equal to 46.4 ± 3.5%) by PKI (lane 3, FIGURE 7A, P = 0.711) but the addition of YK 3-237 restored phosphotyrosine concentrations (106.2 ± 25.6%) to control values (lane 4, P > 0.999). By contrast, when sAC was inhibited by LRE1 (66.3 ± 11.5%, P > 0.999), the YK 3-237 activator increased phosphotyrosine concentrations (lane 6) to values higher (164.1 ± 30.9%, P = 0.324) than those observed in control conditions (in absence of inhibitors, lane 1). However, YK 3-237-induced phosphotyrosine in the presence of sAC inhibitor did not reach the phosphotyrosine concentrations (164.1 ± 30.9%) observed in human spermatozoa incubated with YK 3-237 alone (238.9 ± 40.4%) (lane 2), but no statistical differences were found between the two treatments (P = 0.143).

YK 3-237 increases human sperm responsiveness to a Ca^{2+} ionophore challenge

Following the guidelines from the WHO (*World Health Organization, 2010*), the responsiveness of human spermatozoa to

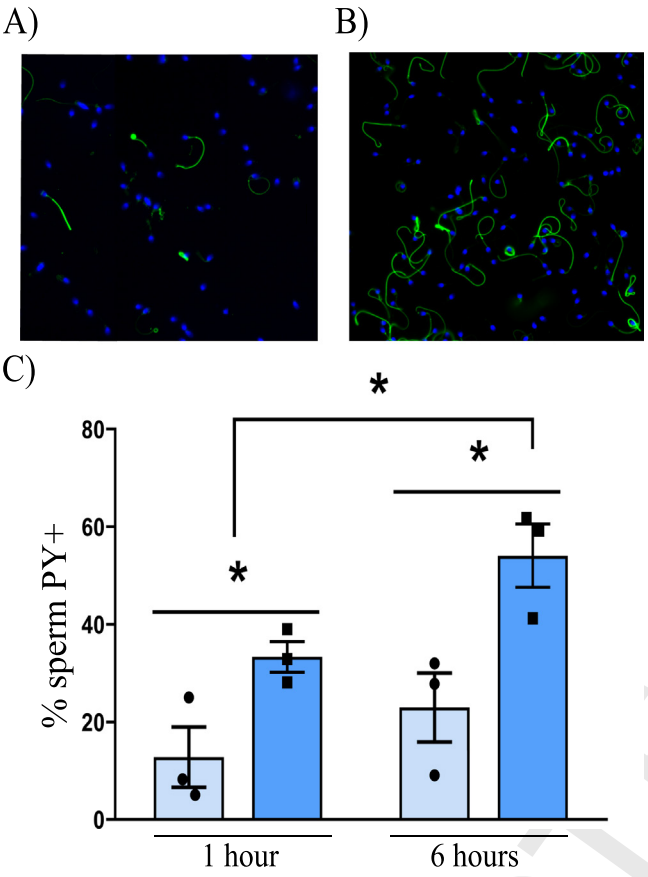

FIGURE 5 Immunolocalization of the YK 3-237-induced tyrosine phosphorylation in human spermatozoa. Human spermatozoa were incubated for up to 6 h at 37°C in capacitating conditions (BWW-modified medium supplemented with HCO_3^- 25 mmol/l and BSA 26 mg/ml) in absence (A) or presence (B) of YK 3-237 (10 µmol/l). (A and B) Representative immunofluorescence images for tyrosine phosphorylation (green) in human spermatozoa incubated for 1 h in absence (A) or in presence (B) of YK 3-237. Sperm nuclei were labelled with DAPI (blue). (C) Quantification of the percentage of spermatozoa showing positive staining for tyrosine phosphorylation in absence (light blue histograms) or presence (dark blue histograms) of YK 3-237 (n = 3). Analysis of variance post-hoc test was used to analyse differences between treatments. When statistical differences were found, the P-values are reported in the graphic. Scale bar in white: 10 µm. BSA = bovine serum albumin; BWW = Biggers–Whitten–Whittingham; DAPI = 4,6-diamidino-2-phenylindole; PY = phosphotyrosine.

undergo acrosome reaction triggered by Ca^{2+} A23187 ionophore was determined (*Aitken et al., 1993*). As depicted in FIGURE 8, both populations, control and YK 3-237-treated spermatozoa responded to Ca^{2+} ionophore by significantly increasing the percentage of live acrosome-reacted spermatozoa (PNA^+/PI^-). Nevertheless, the responsiveness was augmented in spermatozoa incubated in the presence of YK 3-237 in comparison to those incubated in its absence ($P < 0.0001$); 45.44 ± 6.57% versus 26.06 ± 4.98%, respectively (FIGURE 8).

YK 3-237 induces changes in motility parameters associated with hypermotility

Different motility parameters analysed by CASA are summarized in TABLE 1. No significant differences were found in the majority of the motility parameters, either by incubation period (1 versus 6 h) or by treatment (with or without YK 3-237). However, as shown in FIGURE 9, the LIN, STR and WOB indexes were significantly reduced by the YK 3-237 treatment. Specifically, linearity (LIN) in spermatozoa was significantly reduced after 1 h of YK 3-237 incubation (46.70 ± 3.50%; P = 0.002), compared to spermatozoa incubated for 1 h (62.40 ± 4.20%) and 6 h without YK 3-237 (61.16 ± 3.70%; P = 0.008). YK 3-237 also reduced the straightness (STR) of sperm trajectories. STR values of spermatozoa treated with YK 3-237 for 1 h were significantly reduced compared to any group (control or YK 3-237), regardless of the incubation time. Moreover, YK 3-237 treatment reduced significantly WOB values after 1 h in comparison to those spermatozoa incubated without YK 3-237 after 1 h (P = 0.003) and 6 h (P = 0.049) (FIGURE 9). Finally, ALH values were increased by YK 3-237 (3.57 ± 0.26) after 1 h (P = 0.045) of incubation compared to control (2.69 ± 0.15).

YK 3-237 does not affect human sperm plasma membrane integrity and mitochondria membrane activity status

Several sperm parameters related to sperm physiology were determined after 1 h and 6 h of incubation in capacitating conditions (Supplementary Figure 2). YK 3-237 did not have a negative effect on sperm viability (Supplementary Figure 2A), or on acrosome plasma membrane integrity (Supplementary Figure 2B). Sperm mitochondria activity status, determined by the fluorescent probes JC-1 and MitoSOX, was not modified by the presence of YK 3-237 at any time (Supplementary Figure 2C and 2D). Finally, to evaluate whether YK 3-237 (10 µmol/l) induces oxidative damage, lipid peroxidation (determined by concentrations of the aldehyde 4-hydroxynonenal, 4-HNE) was evaluated after 6 h of incubation in the presence or absence of this activator. No differences in the amount of lipid peroxidation product (4-HNE) were observed between YK 3-237 treated and non-treated human spermatozoa (Supplementary Figure 2E and 2F).

DISCUSSION

Sirtuins are a conserved family of deacetylases working as an energy sensor (NAD^+ concentrations) in cells. Sirtuins have an important role in reproductive processes. For instance, SIRT1 is necessary for spermatogenesis (*Coussens et al., 2008*), being present in mouse testes and spermatogonia development phases, but lacking in spermatocytes and spermatids (*Bell et al., 2014*). SIRT1 has been implicated in the biogenesis of the acrosome during spermiogenesis by regulating autophagy (*Liu et al., 2017*). Moreover, the antioxidant role of sirtuins present in human seminal plasma has recently been described. Lower concentrations of SIRT1 in the seminal plasma of oligo-asthenoteratozoospermic and asthenoteratozoospermic men were associated with higher sperm oxidative stress, DNA fragmentation and lipid

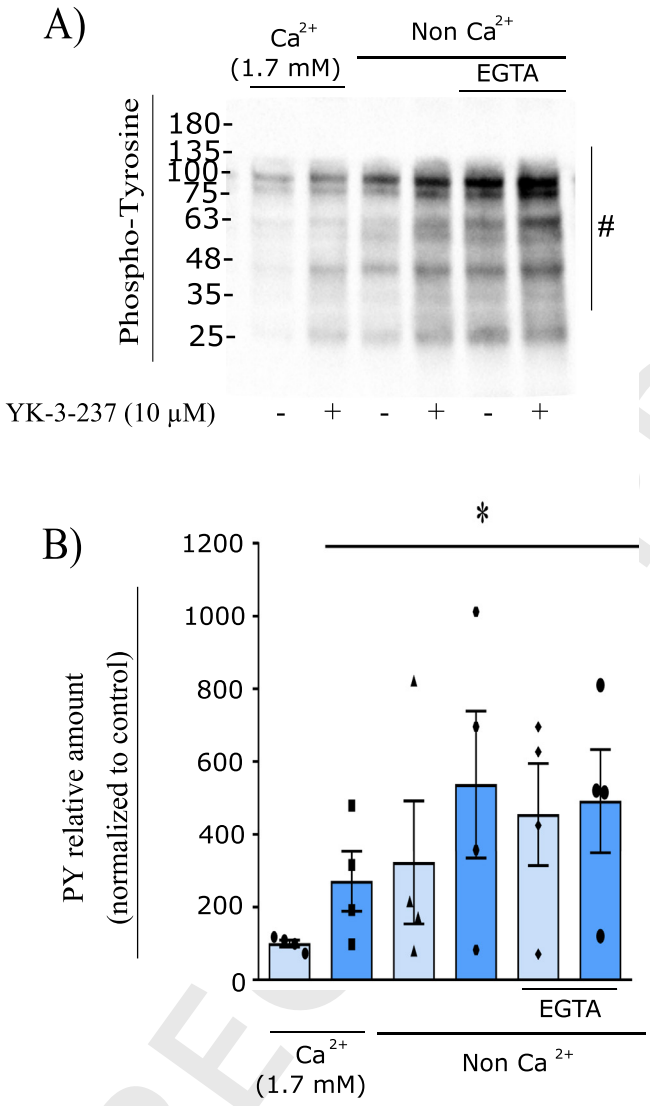

FIGURE 6 YK 3-237-induced tyrosine phosphorylation does not require extracellular calcium. Human spermatozoa were incubated for 6 h at 37°C in capacitating conditions (BWW-modified medium supplemented with HCO_3^- 25 mmol/l and BSA 26 mg/ml) in the presence (1.7 mmol/l) or absence of Ca^{2+} (nominal zero), EGTA (1 mmol/l) or YK 3-237 (10 µmol/l) as indicated. (A) A representative western blot using anti-phosphotyrosine antibody of human spermatozoa is shown. (B) Western blots were analysed using Image Lab (n = 3). Light blue bars represent control conditions; dark blue bars represent YK 3-237 treatment. Bars represent the average ± SEM. For comparison between blots, pixels for each lane contained in the region marked by # (proteins in a 35–135 kDa range) were quantified and normalized using the control (Ca^{2+} 1.7 mmol/l) lane as reference (100%). Analysis of variance post-hoc test was used to analyse differences from control (lane 1 = capacitating conditions in presence of Ca^{2+}). The P-value of each comparison is as follows: control versus control + YK 3-237, P = 0.041; control versus capacitating conditions in absence of Ca^{2+}, P = 0.039; control versus capacitating conditions in absence of Ca^{2+} + YK 3-237, P = 0.0283; control versus capacitating conditions + EGTA, P = 0.0123; control versus capacitating conditions + EGTA + YK 3-237, P = 0.0137. µM = µmol/l; mM = mmol/l. BSA = bovine serum albumin; BWW = Biggers–Whitten–Whittingham; CAP = capacitating medium; PY = phosphotyrosine.

peroxidation, leading to immotile and immature spermatozoa (*Mostafa et al., 2018*; *2020*; *Nasiri et al., 2021*). The current results have shown that SIRT1 is localized mainly in the connection piece of mature human spermatozoa.

In the screening for molecular targets that might affect human spermatozoa features through sirtuin pathways in this study, YK 3-237, a SIRT1 activator, was used, previously used to induce the activation of renal fibroblasts *in vitro* (*Ponnusamy et al., 2015*) and to inhibit the proliferation of triple-negative breast cancer cells (*Yi et al., 2013*) through the SIRT1 pathway. It was found that YK 3-237 enhances human sperm events, such as capacitation-related processes like tyrosine phosphorylation, but surprisingly in a sperm protein acetylation-independent manner. Acetylated-lysine is found along the sperm tail, as described previously in human spermatozoa (*Sun et al., 2014*), with a reduced number of spermatozoa also showing fluorescence in the equatorial segment of the head. The results showed the levels of acetylation on lysine residues detected by western blotting were not modified by YK 3-237 within the time frame studied. Interestingly, an intense acetylation was observed in human sperm proteins around 50 kDa, probably corresponding to the acetylation of tubulin, as described previously in

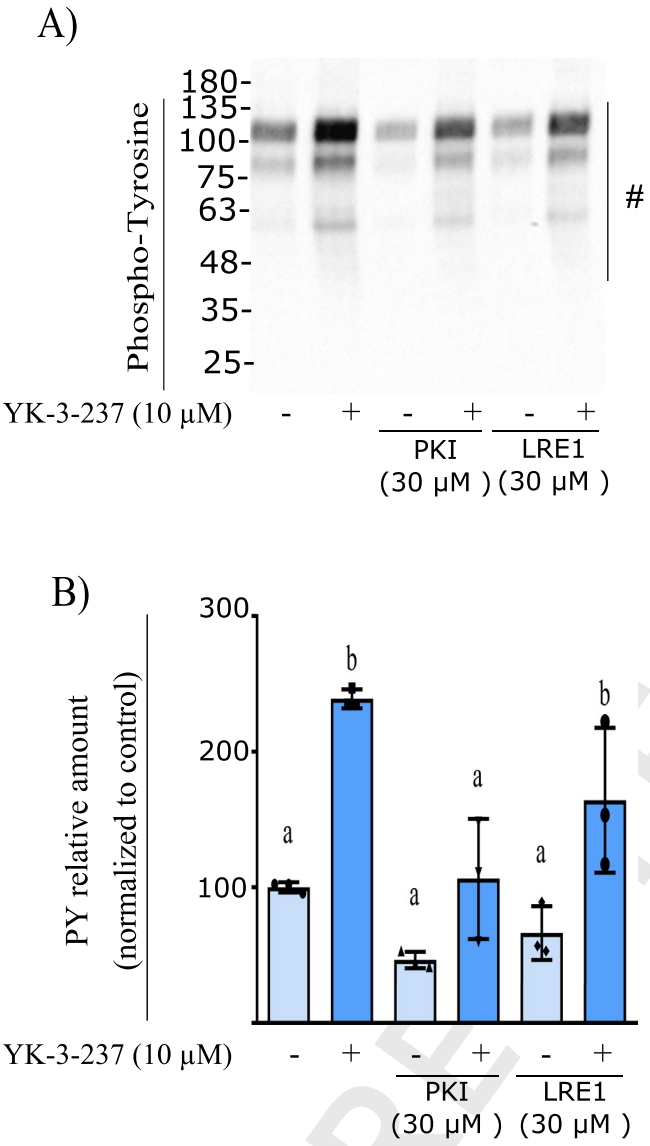

FIGURE 7 YK 3-237 acts downstream of the sAC/PKA pathway in human spermatozoa. (A) A representative western blot using anti-phosphotyrosine antibody of human spermatozoa incubated for 6 h at 37°C in indicated conditions in presence or absence of YK 3-237 (10 µmol/l); the sAC inhibitor LRE1 (30 µmol/l) and PKA inhibitor PKI (30 µmol/l) is shown. (B) Western blots were analysed using Image Lab (n = 3). Bars represent the average ± SEM. For comparison between blots, pixels for each lane contained in the region marked by # in (A) (proteins in a 35–135 kDa range) were quantified and normalized using the control (HCO_3^- 25 mmol/l and BSA 26 mg/ml) lane as reference (100%). Analysis of variance post-hoc test was used to analyse differences with control (lane 1). When statistical differences were found, the P-values are reported in the graphic. µM = µmol/l. BSA = bovine serum albumin; PY = phosphotyrosine.

mouse spermatozoa (*Ritagliati et al., 2018*). This finding suggests that either (i) SIRT1 is not the major isoform enzyme leading to protein lysine acetylation or (ii) that there is a strict regulation of the redundancy of sirtuin isoform activity in human spermatozoa. In this regard, it cannot be ruled out that other sirtuins, such as SIRT3 or SIRT6, which have been recently described in human spermatozoa (*Ritagliati et al., 2018*), might counterbalance the over-activation of SIRT1 by YK 3-237. In addition to these suggestions, it is postulated that SIRT1 could be degraded in human spermatozoa during the process of maturation, as previously described in the mouse during spermatogenesis (*Bell et al., 2014*). This possible SIRT1 degradation could result in residual levels of SIRT1 on a tiny specific region of the connecting piece of human spermatozoa. Future experiments using human testis biopsy might confirm this idea.

This study shows that YK 3-237 exerts its effect on human sperm capacitation-related events such as tyrosine phosphorylation, regardless of the protein acetylation status. YK 3-237 increases the levels of protein tyrosine phosphorylation in a bicarbonate- and calcium-dependent manner by three-fold levels, two components of the sperm capacitating medium. Although co-incubation of BSA and YK 3-237 does not modify phosphotyrosine concentrations in human spermatozoa, co-incubation of YK 3-237 in the presence of BSA plus bicarbonate induces the maximum phosphotyrosine concentrations. Regarding sAC/PKA signalling in spermatozoa, it is well known that Ca^{2+} and bicarbonate act as stimulators of the sperm-soluble adenyl cyclase (sAC) that increases cAMP concentrations, which eventually activates PKA, leading to the phosphorylation of sperm downstream proteins (*Chen et al., 2000*; *Jaiswal and Conti, 2003*; *Litvin et al., 2003*). YK 3-237 was able to partially overcome the inhibitory effect on phosphotyrosine via LRE1 and PKI inhibitors of sAC and PKA, respectively. These results suggest that YK 3-237 might work downstream of sAC in a bicarbonate- and calcium-dependent way. However, a possible direct effect of YK 3-237 on human sperm phosphodiesterases cannot be ruled out, which would inhibit its enzymatic activity, subsequently leading to an increase in cAMP concentrations. A second mechanism may act in parallel through the sAC/PKA pathway, so that YK 3-237 could perhaps work in another signalling pathway independent of PKA. Ca^{2+} has a biphasic role in the spermatozoa of several species (*Baker et al., 2004*; *González-Fernández et al., 2012*; *Marín-Briggiler et al., 2003*; *Navarrete et al., 2015*). By evaluating the status of human spermatozoa incubated in nominal zero calcium or in EGTA capacitating medium (containing bicarbonate and BSA) (*Baker et al., 2004*; *Battistone et al., 2014*), phosphotyrosine concentrations also increased in the absence of calcium. Interestingly, the effects of YK 3-237 only increase sperm phosphotyrosine concentrations in calcium control (1.7 mmol/l) and calcium nominal zero medium, but not in the presence of EGTA. These data allow us to ascertain that in order for YK 3-237 to act it is necessary for the medium to contain residual Ca^{2+} content to promote the phosphotyrosine in human sperm proteins.

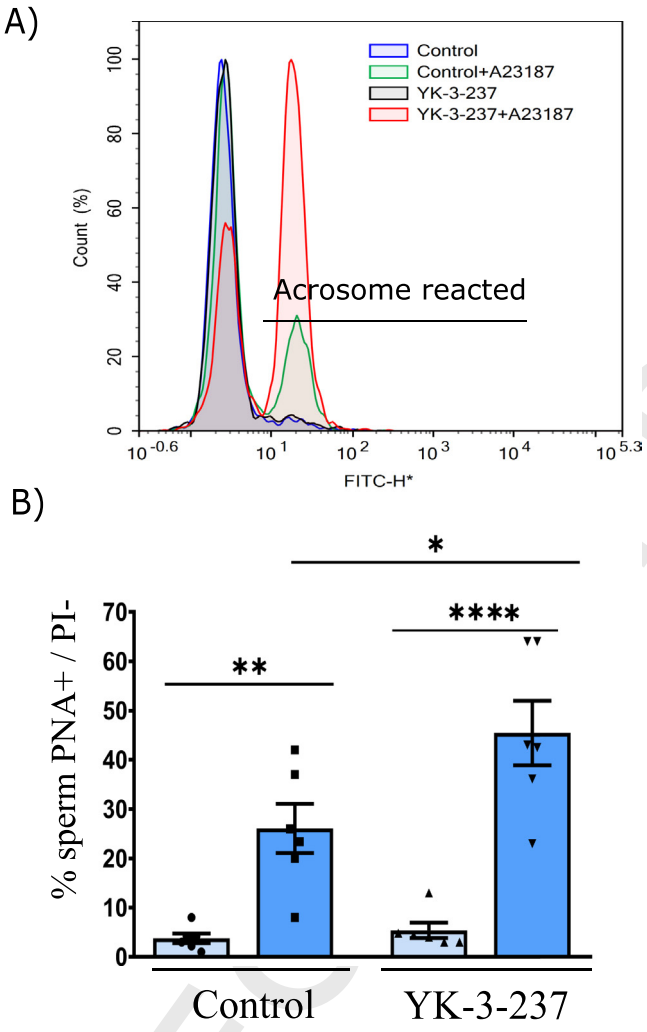

FIGURE 8 YK 3-237 enhances human spermatozoa responsiveness to the calcium ionophore (A23187) challenge. Human spermatozoa were incubated in BWW medium in presence or absence of YK 3-237 (10 µmol/l) for 2 h at 37°C. Later on, half of the spermatozoa were subjected to the Ca^{2+} ionophore (A23187) challenge following the World Health Organization recommendations; the other half acted as control. Briefly, spermatozoa were incubated with A23187 (10 µmol/l) for 15 min. Then, calcium ionophore was eliminated by washing the spermatozoa with BWW. (A) A representative cytogram of a complete experimental design is displayed: control without A23187 (blue); control plus A23187 (green); YK 3-237 without A23187 (black) and YK 3-237 plus A23187 (red). Two sperm populations of live spermatozoa (PI^-) are shown: one containing acrosome-reacted spermatozoa alive (PNA-$FITC^+$) and the other one with the acrosome intact (PNA-$FITC^-$). (B) Histograms represent the percentage of live spermatozoa (PI^-) and acrosome-reacted (PNA-$FITC^+$). Light blue bars represent control conditions whereas dark blue bars represent A23187 treatment. Values represent the mean ± SEM of six independent experiments. Data were analysed statistically by one-way analysis of variance. When statistical differences were found, the *P*-values are reported in the graphic. BWW = Biggers–Whitten–Whittingham.

TABLE 1 THE EFFECT OF YK 3-237 (10 µMOL/L) ON HUMAN SPERM MOTILITY PARAMETERS DETERMINED BY CASA

Time (h)	**1**		**6**	
Treatment	**Control**	**YK 3-237 (10 µmol/l)**	**Control**	**YK 3-237 (10 µmol/l)**
Motile (%)	53.99 ± 7.02	56.60 ± 6.61	58.21 ± 5.88	53.27 ± 6.23
Motile no progressive (%)	10.88 ± 1.59	18.91 ± 1.59	11.20 ± 1.41	10.65 ± 1.04
Motile progressive (%)	43.11 ± 6.34	37.69 ± 5.49	47.01 ± 5.56	42.61 ± 5.75
VCL (µmol/l)	82.42 ± 4.95	96.50 ± 5.75	96.85 ± 6.15	85.57 ± 5.64
VSL (µmol/l)	52.40 ± 4.31	44.54 ± 3.07	58.76 ± 4.32	54.43 ± 3.66
VAP (µmol/l)	55.45 ± 4.07	51.50 ± 2.60	61.95 ± 4.17	57.87 ± 3.56
BCF (Hz)	10.44 ± 0.31	10.18 ± 0.28	10.96 ± 0.47	11.21 ± 0.44

Values are expressed as the mean ± SEM. No statistical differences were found.

BCF = beat cross frequency; CASA = computer-assisted sperm analysis; VAP = average path velocity; VCL = curvilinear velocity; VSL = straight line velocity.

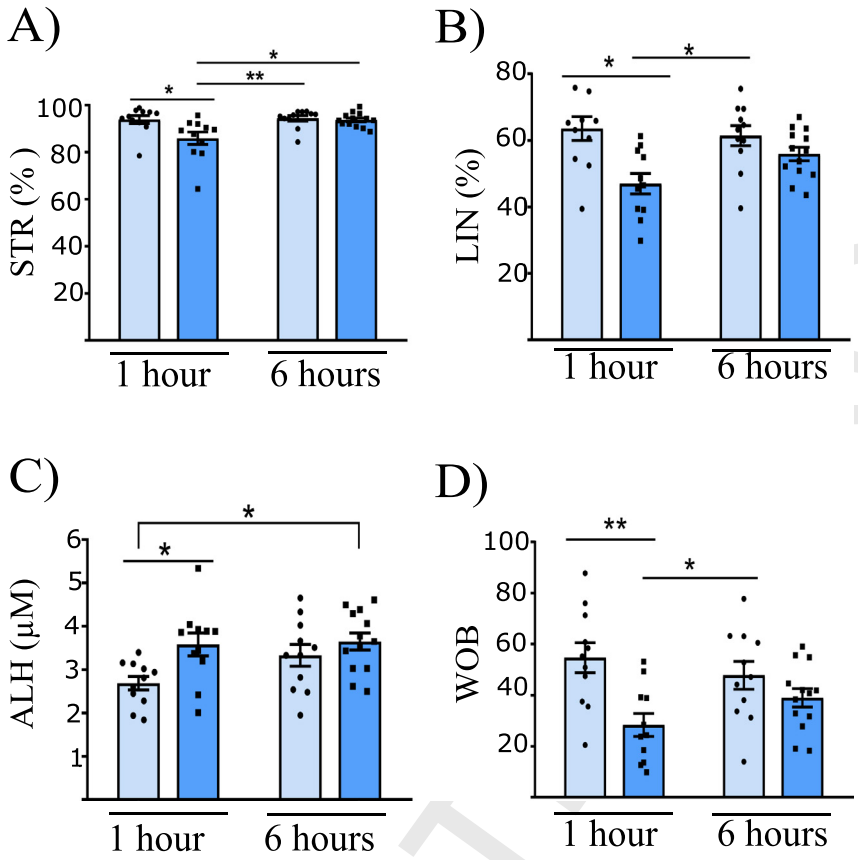

FIGURE 9 Effect of YK 3-237 on several human sperm motility parameters. Human spermatozoa were incubated for up to 6 h at 37°C in capacitating conditions (BWW-modified medium supplemented with HCO_3^- 25 mmol/l and BSA 26 mg/ml) in presence or absence of YK 3-237 (10 µmol/l). Measurement of human sperm motility was performed after 1 h and 6 h. (A) Histograms represent the percentage of spermatozoa showing straightness (STR) trajectory. (B) Histograms represent the percentage of spermatozoa showing linear (LIN) trajectory. (C) Histograms represent the amplitude of lateral sperm head displacement (ALH) value. (D) Histograms represent the wobble movement coefficient (WOB). Light blue bars represent control conditions whereas dark blue bars represent YK 3-237 treatment. Values represent the mean ± SEM of 11 independent experiments. Data were analysed statistically by one-way analysis of variance. When statistical differences were found, the *P*-values are reported in the graphic. µM = µmol/l. BSA = bovine serum albumin; BWW = Biggers–Whitten–Whittingham.

Sperm capacitation status has also been related to the ability of the spermatozoa to undergo the acrosome reaction when stimulated by calcium ionophore (*Aitken et al., 1993*). Importantly, the calcium ionophore-induced acrosome reaction has been correlated with IVF rates (*Liu and Baker, 1998*). Human spermatozoa incubated in the presence of YK 3-237 augmented their responsiveness to the ionophore challenge significantly (by two times). However, the acrosome membrane integrity of spermatozoa not stimulated by A23187 is unaffected by the activator. In addition, YK 3-237 did not affect intracellular mechanisms that compromise human sperm viability, mitochondrial activity status or lipid peroxidation. Although YK 3-237 did not affect the percentage of motile spermatozoa or their kinetic parameters, it induced a motility pattern associated with hypermotility, such as a decrease in the linearity of the sperm trajectory (STR and LIN indexes) and a higher amplitude lateral head displacement (ALH).

In brief, YK 3-237 induces capacitation-related events in human spermatozoa such as raising the levels of protein tyrosine phosphorylation and increasing the percentage of spermatozoa that undergo the acrosome reaction after the ionophore challenge. YK 3-237 probably acts on human spermatozoa by a mechanism independent of protein lysine acetylation, but dependent on bicarbonate and calcium. Further research is needed to establish the exact mechanism of YK 3-237 on human sperm capacitation-related events.

UNCITED REFERENCE

Calle-Guisado et al., 2019

ACKNOWLEDGEMENTS

Thanks to Darya A Tourzani for her help with correcting the English grammar in the manuscript. David Martin-Hidalgo was the recipient of a postdoctoral fellowship from Junta de Extremadura and European Social Funds (PO17020) and is currently supported by 'Stop Fuga de Cerebros', funded by Roche Farma SA and implemented by FundeSalud Extremadura (FS P20-34). Lauro González-Fernández is granted by RYC2020-028915-I funded by MCIN/AEI/10.13039/50110 0 011033 and by 'ESF Investing in your Future'. This work was supported by 'Fundação para a Ciência e a Tecnologia' – FCT, co-funded by FEDER through the COMPETE/QREN, FSE/POPH and POCI – COMPETE 2020 (POCI-01-0145-FEDER-007491) funds to Marco G Alves (IFCT2015 and PTDC/MEC-AND/28691/2017), UMIB (FCT

UID/Multi/00215/2019), and QOPNA (FCT UID/QUI/00062/2019). Pedro F Oliveira is funded by national funds through FCT – Fundação para a Ciência e a Tecnologia, IP, under the Scientific Employment Stimulus – Institutional Call – reference CEECINST/00026/2018. This work was also supported by the Regional Government of Extremadura, Spain (grants: IB20154 and GR18094).

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2022.07.011.

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Received 21 March 2022; received in revised form 7 July 2022; accepted 14 July 2022.