Manipulation of bicarbonate concentration in sperm capacitation media improves in vitro fertilisation output in porcine species

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Abstract

Background: The in vivo concentration of bicarbonate (HCO$_3^-$), one of the essential sperm capacitating effectors, varies greatly in the different environments sperm go through from cauda epididymis to the fertilisation site. On the contrary, porcine in vitro sperm capacitation and fertilisation media usually contains a standard concentration of 25 mmol/L, and one of the main problems presented is the unacceptable high incidence of polyspermy. This work hypothesised that by modifying the HCO$_3^-$ concentration of the medium, the output of in vitro sperm capacitation and fertilisation could be increased.

Results: Once exposed to the capacitation medium, the intracellular pH (pH$_i$) of spermatozoa increased immediately even at low concentrations of HCO$_3^-$, but only extracellular concentrations of and above 15 mmol/L increased the substrates protein kinase A phosphorylation (pPKAs). Although with a significant delay, 15 mmol/L of HCO$_3^-$ stimulated sperm linear motility and increased other late events in capacitation such as tyrosine phosphorylation (Tyr-P) to levels similar to those obtained with 25 mmol/L. This information allowed the establishment of a new in vitro fertilisation (IVF) system based on the optimization of HCO$_3^-$ concentration to 15 mmol/L, which led to a 25.3% increment of the viable zygotes (8.6% in the standard system vs. 33.9%).

Conclusions: Optimising HCO$_3^-$ concentrations allows for establishing an IVF method that significantly reduced porcine polyspermy and increased the production of viable zygotes. A concentration of 15 mmol/L of HCO$_3^-$ in the medium is sufficient to trigger the in vitro sperm capacitation and increase the fertilisation efficiency in porcine.

Keywords: Adcy10, Boar, Monospermy, PKA

Background

Fertilisation is considered a multifactorial process in which many factors are directly and/or indirectly involved. The success rate of this physiological process depends to a high degree on the ability of spermatozoa to undergo capacitation and penetrate the oocyte vestments [1]. In vivo sperm capacitation is a gradual event that is actively and progressively coordinated within succeeding segments of the female genital tract [2]. The changes occurring in spermatozoa during in vivo capacitation begin as soon as they are ejaculated as a result of, among other reasons, the bicarbonate (HCO$_3^-$) intake, subsequent activation of cAMP synthesis, intracellular pH alkalisation (pH$_i$), increase in intracellular Ca$^{2+}$ concentration and motility activation (fast capacitation events) [3]. Once inside the female genital tract, other events occur in a sequential and parallel way for an extended period, such as hyperactivation, tyrosine phosphorylation (Tyr-P) and preparation to undergo acrosome reaction (AR) (slow capacitation events). Both fast and slow events depend on the HCO$_3^-$ and Ca$^{2+}$ concentrations,
but the slow ones also depend on the presence of cholesterol acceptors like albumin (reviewed by Visconti [3]). The inward transport of $\text{HCO}_3^-$ from the surrounding medium during capacitation involves electrogenic $\text{Na}^+/\text{HCO}_3^-$ co-transporter (NBC) and $\text{Cl}^-/\text{HCO}_3^-$ anion exchangers [4]. It starts with induction of the atypical soluble adenyl cyclase Adcyl10 aka sAC (from now on Adcy10), increase in cAMP levels and activation of protein kinase A (PKA) [5]. Numerous studies have indicated that PKA phosphorylates several substrates (pPKAs) which supposes the initial point either for fast or slow events of the capacitation [3]. In porcine sperm, previous studies suggest that phosphorylation of pPKAs rapidly increases in ~160 s through the action of $\text{HCO}_3^-$, after which the activity increases slowly to reach a maximum at 21 min [6]. The $\text{HCO}_3^-$ acts as a key capacitating agent and its importance in mediating changes in spermatozoa has been demonstrated in multiple studies in mice and humans. Knock-out mice that fail to express Adcy10 were infertile [7, 8] and females with defective $\text{HCO}_3^-$ secretion in the uterus produced low sperm capacitation and fertility [9].

After maturing as they pass through the epididymis, the spermatozoa are stored in the epididymal cauda in low $\text{HCO}_3^-$ conditions (~4 mmol/L [10]). During ejaculation, the spermatozoa come into contact with the seminal plasma, which contains higher $\text{HCO}_3^-$ concentrations (~20 mmol/L) [11] but also decapacitating factors, mainly spermadhesins from accessory sex glands, that coat and stabilise the sperm surface to prevent premature capacitation [12]. Once in the female genital tract, sperm are subjected to different selection mechanisms that remove the immature or damaged ones. As they move forward in the uterus towards the oviduct, the spermatozoa have to swim through several environments increasingly rich in $\text{HCO}_3^-$ (~25 mmol/L [7]) and the decapacitating factors are gradually removed, enabling membrane destabilisation and capacitation. Inside the oviduct, they remain attached to the epithelium after the utero-tubal junction, in the caudal isthmus, establishing the sperm reservoir. Once ovulation occurs, the spermatozoa are gradually released from the sperm reservoir and swim towards the fertilisation site at the ampullary-isthmic junction [13]. However, many of the ovulation signals and mechanisms of gradual sperm release from the reservoir are still unknown, and, in vitro conditions are far from ideal for gradually exposing spermatozoa to capacitating conditions. Currently, most of the in vitro capacitation and fertilisation (IVF) methods, in porcine and other species, expose all the spermatozoa to a fixed $\text{HCO}_3^-$ concentration of 25 mmol/L at the same time [14–19], providing a static IVF system that little resembles the dynamic system of fluids to which gametes are exposed in vivo. Despite the development of chemically defined fertilisation media [15, 20, 21], different spermatozoa and oocytes co-incubation times [22], sperm capacitation methods [23] and the use of innovative IVF methods in straws or in devices in which both gametes are physically separated [24], polyspermy, one of the major problems in porcine IVF, persists. The microenvironment of in vitro capacitation and fertilisation currently used in laboratories is probably far from optimal for porcine species. The objective of this work was to study the effect of different concentrations of $\text{HCO}_3^-$ on sperm capacitation, mimicking as far as possible the increase that takes place in vivo. For this purpose, different $\text{HCO}_3^-$ concentrations were tested on PKAs activation, pH and sperm motility, and on several subsequent events downstream of PKA activation in the cascade of capacitation, such as Tyr-P and the AR. Finally, the in vitro fertility parameters were studied. The results of this study could increase our knowledge of sperm physiology and lead to an improvement of in vitro sperm capacitation and oocyte fertilisation output in porcine species.

**Material and methods**

**Spermatozoa collection and preparation**

Epididymes from mature Large White boars (*Sus scrofa*) were obtained from a slaughterhouse and transported to the laboratory within 30 min of slaughter. The cauda epididymis was dissected and the epididymal duct was isolated. A 24G BD Insyte™ catheter (381212, Becton Dickinson Infusion Therapy Systems, Inc., Sandy, Utah, USA) adapted to a syringe was inserted into the epididymal duct, and sperm samples were obtained by pressurised air through the deferens duct. Spermatozoa samples were incubated for up to 60 min (1, 5, 15, 30 and 60 min, depending on the experiment) in modified Tyrode’s albumin lactate pyruvate media (TALP) based on that described by Rath et al. [15], composed by 114.06 mmol/L NaCl, 3.20 mmol/L KCl, 0.50 mmol/L MgCl$_2$·6H$_2$O, 18.00 mmol/L sodium lactate, 0.35 mmol/L NaH$_2$PO$_4$·H$_2$O, 5.00 mmol/L glucose, 2.00 mmol/L caffeine, 8.00 mmol/L calcium lactate·5H$_2$O, 1 mg/mL polyvinyl alcohol, 0.17 mmol/L kanamycin and 0.003 mmol/L phenol red, 3 mg/mL bovine serum albumin (BSA), 1 mmol/L sodium pyruvate, but containing different $\text{HCO}_3^-$ concentrations: 0, 5, 15 or 25 mmol/L. Prior to sperm incubation, all media were adjusted to pH 7.4. The pH of the medium containing 0 mmol/L was adjusted using NaOH and HCl, the pH of the media containing 5, 15 and 25 mmol/L was adjusted in an incubator for nearly 3 h at 38.5 °C, saturated humidity and 1.0%, 3.0% or 5.0% of CO$_2$ atmosphere in air, respectively, according to the Henderson-Hasselbalch equation. A group of spermatozoa incubated in phosphate buffer
saline (PBS) was included as non-capacitating control (NCAP).

**Western blotting (WB)**
Isolated proteins from 1 × 10^6 spermatozoa samples were obtained as described by Navarrete et al. [25]. Briefly, after incubation for up to 60 min in TALP containing 0, 5, 15 or 25 mmol/L of HCO₃⁻ or in NCAP medium, samples were centrifuged and washed in 500 μL PBS. The supernatant was removed, 10 μL Laemmli buffer [26] was added to the pellet, boiled for 5 min and centrifuged once more. Supernatant was supplemented with 5% β-mercaptoethanol, boiled for 3 min, loaded in SDS-PAGE gels (ref. 4561044, BioRad, Hercules, CA, USA) and electro-transferred to 0.45 μm PVDF membranes (Merk KGaA, Darmstadt, Germany). Membranes were first blocked with 5% BSA (A9647, Sigma-Aldrich®, Madrid, Spain) in TBS containing 0.1% Tween 20 (P1379, Sigma-Aldrich®, Madrid, Spain) (A9647, Sigma-Aldrich®, Madrid, Spain) in TBS containing 0.1% Tween 20 (P1379, Sigma-Aldrich®, Madrid, Spain) (T-TBS) and then incubated with the primary polyclonal antibodies anti-phospho-PKA substrates (9624, Cell Signal-}

Spermatozoa intracellular pH (pHi)
The analysis of pHi was carried out in spermatozoa (30 × 10⁶ cells/mL) loaded with 5 μmol/L of the pH-sensitive dye BCECF-AM (B1150, Sigma-Aldrich®, Madrid, Spain) for 30 min at 38.5 °C. After that, the samples were centrifuged at 700 g for 3 min to remove the excess of dye and resuspended in PBS without Ca²⁺ and Mg²⁺ and incubated again for 15 min at 38.5 °C for the de-esterification of the dye. Finally, the samples were centrifuged and resuspended in NCAP, 0 mmol/L, 5 mmol/L, 15 mmol/L and 25 mmol/L of HCO₃⁻ for 1 and 60 min. The fluorescence was monitored using a spectrofluorometer (FP-6300, Jasco®, Cremella, Italy) every 2 s for a total time of 300 s. A calibration of the system was first performed using BCECF-AM stained and equilibrated spermatozoa at pH 6.0, 6.5, 7.0, 7.5 and 8.0 in the presence of 0.1% Triton X-100 by adjusting the pH with HCl and NaOH [27]. The emitted fluorescence ratio from the excitation at 490/440 nm was calculated and the regression line for extracellular pH (pHe) vs. the 490/440 nm ratio was obtained (Additional file 1). The pH of sperm cells was estimated from the regression line.

Spermatozoa motility
Sperm motion assay was carried out using a computer-assisted spermatozoa motility analysis (CASA), the ISAS® system (PROISER R + D S.L., Valencia, Spain), connected to a negative phase-contrast microscope (magnification ×100; Leica DMR, Wetzlar, Germany) and a digital camera (Basler Vision, Ahrensburg, Germany). Spermatozoa motility was determined after 1, 15, 30 and 60 min of incubation in the different TALP media previously described. For this purpose, 4 μL of samples were placed on a warmed (38.5 °C) 20-μm SpermTrack® chamber (STP-21006, PROISER R + D S.L., Valencia, Spain) and motility parameters were determined into 3 different fields per sample and time of incubation: the percentage of total motile spermatozoa (Mot, %), motile progressive spermatozoa (MotPro, %), curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity of the curvilinear trajectory (LIN, ratio of VSL/VCL, %), straightness (STR, ratio of VSL/VAP, %), wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL, %), amplitude of lateral head displacement (ALH, μm) and beat cross-frequency (BCF, Hz). Moreover, the spermatozoa from each replicate were classified in 3 different clusters per experimental group and time of incubation to identify groups of spermatozoa with a high degree of similarity in their motility pattern: i) Cluster 1: spermatozoa with the poorest motility (lowest values in all CASA parameters); ii) Cluster 2: spermatozoa with the most linear trajectory (high values of VSL, LIN, STR and WOB); and iii) Cluster 3: spermatozoa with the most curvilinear trajectory (high values of VCL and ALH and low values of VSL, LIN and STR) (Additional file 2).

Spermatozoa tyrosine phosphorylation (Tyr-P)
Indirect immunofluorescence (IF) was performed as described by Luño et al. [28] to study sperm Tyr-P. Samples processing was carried out at 4 °C. After 60 min of incubation in TALP media containing different HCO₃⁻ concentrations, sperm samples were first washed with PBS and centrifuged at 270×g for 10 min and then fixed in 2% p-formaldehyde/PBS for 60 min. The sample blocking was carried out overnight in a wet chamber in 2% (w/v) BSA in PBS. After that, samples were re-centrifuged at 270×g for 10 min, resuspended in PBS and spread on glass slides. The primary antibody used for incubation was anti-phosphotyrosine (4G10, Millipore, CA, USA, 1:300 in 0.1% BSA in PBS). The slides were washed with PBS and then incubated with the secondary antibody, fluorescein-conjugated goat anti-mouse
In vitro fertilisation (IVF)

Porcine oocytes were isolated from ovaries obtained from 6- to 7-month-old animals at a local slaughterhouse and transported to the laboratory in saline solution at 38.5 °C. Once in the laboratory, the ovaries were washed in 0.04% cetrimonide solution (w/v) and saline solution. Cumulus-oocytes complexes (COCs) were collected from antral follicles (3–6 mm diameter) and washed twice with Dulbecco’s PBS. Groups of 50 COCs with complete and dense cumulus oophorus were in vitro matured in 500 μL NCSU-37 medium based on that described by Petters and Wells [29] composed by 108.73 mmol/L NaCl, 25 mmol/L NaHCO₃, 4.78 mmol/L KCl, 1.19 mmol/L KH₂PO₄, 1.19 mmol/L MgSO₄·7H₂O, 1.70 mmol/L CaCl₂·2H₂O, 5.55 mmol/L glucose, 1.00 mmol/L glutamine, 12.00 mmol/L sorbitol, 0.18 mmol/L penicillin and 0.07 mmol/L streptomycin, for 42 h, as previously described by Funahashi et al. [30]. After IVM, oocytes were washed twice with Dulbecco’s PBS. Groups of 50 COCs were collected from antral follicles (3–6 mm diameter) and washed twice with Dulbecco’s PBS. After 18 h of co-culture, putative zygotes were fixed and IVF parameters were evaluated under an epifluorescence microscope at × 400 (Leica® DMR, USA) as previously described by Matás et al. [31]: the percentage of penetrated oocytes (Pen, %), percentage of monospermy of penetrated oocytes (Mon, %), number of spermatozoa penetrating each oocyte (SPZ/O), number of sperm bound to ZP (SPZ/ZP) and efficiency (%) which represents the final number of putative zygotes in each group per 100 penetrated oocytes.

Statistical analysis

All statistical analyses were performed using IBM SPSS v.19 (SPSS Inc. Chicago, IL, USA) and data were analysed by one-way ANOVA. In the motility experiment, 2 types of analysis were carried out per field: i) data were analysed using the general linear repeated-measures procedure, and ii) data were grouped by a non-hierarchical k-means clustering for each time of incubation and a repeated-measures procedure was followed for each cluster. Three different clusters centers where established per motility parameter to classify each spermatozoa (Additional file 2). In both sets of analyses, the obtained results were compared by one-way ANOVA. Data are expressed as the mean ± standard error of the mean (SEM). Differences were considered statistically significant at P < 0.05, and when these analyses revealed a significant effect, values were compared using the least significant difference pairwise multiple comparison post-hoc test (Tukey). A statistical tendency was considered when P ≥ 0.05 and ≤ 0.75.

Results

Effect of HCO₃⁻ concentration on spermatozoa functionality

Among the ions existing along the route that spermatozoa must follow from the epididymis, HCO₃⁻ has an important role in two main sperm functions: i) collaborating in the functional maturation of spermatozoa by affecting membrane dynamics and ii) buffering the environment [32–34]. Moreover, HCO₃⁻ is a key factor in inducing sperm capacitation by direct stimulation of Adcy10 before triggering the cascade of fast and slow events of capacitation. Here, the effect of different HCO₃⁻ concentrations on these capacitating events was investigated.

Spermatozoa protein kinase A substrates phosphorylation (pPKAs)

The effect of HCO₃⁻ concentration on pPKAs after 60 min in NCAP, 0 mmol/L, 5 mmol/L, 15 mmol/L and 25 mmol/L groups are shown by WB in Fig. 1. The results...
showed that 15 mmol/L and 25 mmol/L produced a significantly higher degree of pPKAs compared with the rest of the groups \( (P < 0.05) \). Figure 1A showed that low concentrations of \( \text{HCO}_3^- \) (0 and 5 mmol/L) produced low levels of pPKAs, similar to those produced in NCAP group. However, the addition of a higher \( \text{HCO}_3^- \) concentration (of and above 15 mmol/L) induced a significant increment in pPKAs. When comparing experimental replicates, 15 mmol/L and 25 mmol/L produced a significantly higher degree of pPKAs compared with the rest of the groups \( (P < 0.05) \) (Fig. 1B). Although it has been described that the pPKAs is time-dependent, as shown in Fig. 2, it is also concentration-dependent. When low \( \text{HCO}_3^- \) concentrations were used, like 0 or 5 mmol/L, the highest level of pPKAs was achieved since the beginning of incubation (Fig. 2A and C). On the other hand, when higher levels of \( \text{HCO}_3^- \) were used, 15 and 25 mmol/L, pPKAs are progressively increasing (Fig. 2E and G), being faster as \( \text{HCO}_3^- \) concentration is higher (25 mmol/L). Through semi-quantification it could be determined that inexistence or low concentrations of \( \text{HCO}_3^- \) (0 or 5 mmol/L) could not provoke pPKAs similar to control (25 mmol/L) after 60 min of incubation (Fig. 2B and D). Higher concentrations of \( \text{HCO}_3^- \) produce the highest pPKAs in a concentration-dependent way. A \( \text{HCO}_3^- \) concentration of 15 mmol/L produced the highest pPKAs after 60 min of incubation \( (P < 0.05) \) and 25 mmol/L after 15 min \( (P < 0.05) \) (Fig. 2H).

**Spermatozoa intracellular pH (pH)**

Adcy10/cAMP activation and further cascade events (including PKAs phosphorylation) are boosted by the intracellular alkalinisation of spermatozoa associated with the intake of external \( \text{HCO}_3^- \), but the relation with the specific \( \text{HCO}_3^- \) concentration in capacitating conditions is still unclear. The pH of the spermatozoa exposed to NCAP or 0 mmol/L, 5 mmol/L, 15 mmol/L and 25 mmol/L of \( \text{HCO}_3^- \) is shown in Fig. 3. At 1 min of incubation, there were no differences in the pH of spermatozoa exposed to NCAP or 0 mmol/L (NCAP: 6.65 ± 0.03, 0 mmol/L: 6.77 ± 0.01, 5 mmol/L: 6.93 ± 0.02, 15 mmol/L: 7.00 ± 0.03, 25 mmol/L: 7.06 ± 0.05, \( P > 0.05 \)). After 60 min of incubation, the pH of spermatozoa increased in all groups, but only those that contained \( \text{HCO}_3^- \) were statistically higher than NCAP and 0 mmol/L. Higher concentrations of \( \text{HCO}_3^- \) (0 mmol/L: 6.65 ± 0.03, 5 mmol/L: 6.77 ± 0.01, 15 mmol/L: 6.93 ± 0.02, 25 mmol/L: 7.00 ± 0.03, 25 mmol/L: 7.06 ± 0.05, \( P < 0.05 \)).

**Spermatozoa motility**

One of the first events related with Adcy10/PKA pathway activation by \( \text{HCO}_3^- \) is sperm motility stimulation. Thus, the effect of \( \text{HCO}_3^- \) on sperm motility and trajectory was studied in capacitating conditions in 0 mmol/L, 5 mmol/L, 15 mmol/L and 25 mmol/L (Figs. 4, 5 and 6). The results showed that the \( \text{HCO}_3^- \) concentration did not affect Mot (ranged from 84.9% to 92.9%), MotPro (from 83.2% to 92.4%) or VCL (from 138.2 to 165.0 μm/s) but stimulated straight and average path velocities (VSL and VAP, \( P < 0.05 \)) (Fig. 4). A \( \text{HCO}_3^- \) concentration of 15 mmol/L produced significantly higher LIN, STR and WOB than 0 mmol/L and 5 mmol/L but 25 mmol/L did not induce any extra improvement (Fig. 4). There were no statistical differences in ALH and BCF between groups. A visual representation of the effect of \( \text{HCO}_3^- \) on the trajectories of spermatozoa is shown in Fig. 5. An increasingly straight trajectory was directly proportional to the \( \text{HCO}_3^- \) concentration in the medium. Moreover, a cluster analysis revealed that the proportion of spermatozoa with poorer...
Fig. 2 (See legend on next page.)
Motility (cluster 1) was similar between groups, regardless of the HCO$_3^-$ concentration and length of incubation (Fig. 6). The proportion of spermatozoa with the most rectilinear trajectory (cluster 2) was significantly higher ($P < 0.05$) in the 5 mmol/L, 15 mmol/L and 25 mmol/L groups than in 0 mmol/L. In addition, and despite a certain tendency ($P = 0.051$) to decrease in the presence of HCO$_3^-$, there were no statistical differences in the proportion of spermatozoa with the most curvilinear trajectory (cluster 3) between groups.

**Spermatozoa tyrosine phosphorylation (Tyr-P)**

The pH$_i$ seems to favour the cascade of slow and fast events in spermatozoa, including the activation of tyrosine kinases and the subsequent Tyr-P of proteins. In fact, the capacitation status was described to be strongly correlated with Tyr-P [5] as a direct consequence of the action of HCO$_3^-$, whose omission not only inhibits Tyr-P [5], but also diminishes the ability of spermatozoa to bind to ZP [35]. This slow capacitation response must be preceded by lipid scrambling and cholesterol removal by a cholesterol acceptor, normally albumin. Different compartments have been described as undergoing a specific sequence of phosphorylation events during both capacitation and binding to ZP [36]. The present experiment tried to evaluate the effect of HCO$_3^-$ concentration on Tyr-P immunolocation in epididymal porcine spermatozoa (Fig. 7). At 1 min of incubation, all the experimental groups had the same proportion of spermatozoa with patterns I and III (low and high capacitation status, respectively). The 25 mmol/L concentration had a significantly reduced proportion of spermatozoa with pattern II (medium capacitation status) (17.4 ± 3.7%) compared with NCAP (29.2 ± 1.4%) (Fig. 7A). At 60 min of incubation, the proportion of pattern I and II (low and medium capacitation status, respectively) did not show any statistical difference between the media used ($P > 0.05$). However, the proportion of pattern III (high capacitation status) was significantly higher in 15 mmol/L and 25 mmol/L (45.2 ± 3.0% and 45.5 ± 4.3%, respectively; $P < 0.05$) (Fig. 7B). As regards pattern IV, 15 mmol/L and 25 mmol/L produced the highest level of Tyr-P in the spermatozoa tail, which differed significantly from the rest of the groups (68.0 ± 4.7% and 65.9 ± 4.8%, respectively; $P < 0.05$) (Fig. 7C). Taken together, these results showed that a 15 mmol/L concentration of HCO$_3^-$ in the capacitation medium is sufficient to attain a high level of Tyr-P and probably motility activation.

**Fig. 2** Kinetics of different HCO$_3^-$ concentrations on sperm protein kinase A substrates phosphorylation (pPKAs). Sperm were incubated for 1, 5, 15, 30 and 60 min in capacitating medium (TALP) containing 0 mmol/L (A), 5 mmol/L (C), 15 mmol/L (E) and 25 mmol/L (G) of HCO$_3^-$ (4 replicates). In each blot a lane of spermatozoa incubated in PBS was included as non-capacitating control (NCAP). In 0 mmol/L (A) and 5 mmol/L (C) blots a lane for spermatozoa incubated in 25 mmol/L for 60 min was included as positive control. β-actin (β-act) or β-tubulin (β-tub) was used as loading control. Relative amount of semi-quantified signal for 0 mmol/L, 5 mmol/L, 15 mmol/L and 25 mmol/L blots are shown in graphics B, D, F and H, respectively. Results are shown as mean ± SEM. Different letters (a-c) indicate statistically significant differences ($P < 0.05$) between groups.

**Fig. 3** Effect of HCO$_3^-$ on boar epididymal sperm intracellular pH (pH$_i$). Samples were loaded with 5 μmol/L of the pH-sensitive dye BCECF-AM for 30 min at 38.5°C, centrifuged at 700xg for 3 min to remove the excess of dye and resuspended in PBS without Ca$^{2+}$ and Mg$^{2+}$ and incubated again for 15 min at 38.5°C for the de-esterification of the dye. After that, sperm were incubated for 1 and 60 min in capacitating medium (TALP) containing different concentrations of HCO$_3^-$ (0 mmol/L, 5 mmol/L, 15 mmol/L and 25 mmol/L) and non-capacitating medium (NCAP) in 4 replicates. The fluorescence was monitored using a spectrophotometer every 2 s for a total time of 300 s. The emitted fluorescence ratio from the excitation at 490/440 nm was calculated and the regression line for pH$_i$ vs. the 490/440 nm ratio was obtained (Additional file 1). The pH$_i$ of sperm cells was estimated from the regression line. Results are shown as mean ± SEM. Different letters (a, b) in the same time of incubation indicate statistically significant differences ($P < 0.05$).
Effect of $\text{HCO}_3^-$ concentration on IVF
As described above, the concentration of $\text{HCO}_3^-$ affects sperm functionality and modulates sperm capacitation, a regulatory event that prepares spermatozoa for fertilization prior to encountering the oocyte. Based on this, the present study tried to determine the fertility of spermatozoa and the interaction between gametes in the same conditions.

**Monophasic IVF**
The IVF output for penetration, monospermy, SPZ/O, SPZ/ZP and efficiency are shown in Table 1A. In the absence of $\text{HCO}_3^-$ (0 mmol/L) no oocyte was penetrated, which confirms that $\text{HCO}_3^-$ is an indispensable component of the IVF medium for the spermatozoa to bind to ZP and to penetrate the oocyte. When 5 mmol/L was used, all the studied parameters were significantly lower than when 15 mmol/L or 25 mmol/L was used. The 25 mmol/L concentration had a lower efficiency (8.6 ± 1.7%) than 15 mmol/L because, although penetration was high, monospermy was low. The use of 15 mmol/L significantly increased the efficiency of the technique to 26.2 ± 3.1%, with a lower penetration but a higher monospermy ($P < 0.05$) than at 25 mmol/L. In light of these results, it is clear that sperm fertility was affected by the mere presence of $\text{HCO}_3^-$ and also its concentration. This study then turned to the task of identifying an IVF method that increased the efficiency of obtaining potentially viable zygotes by optimizing the $\text{HCO}_3^-$ concentration to which gametes are gradually exposed.

**Biphasic IVF (spermatozoa pre-incubation + monophasic IVF)**
The pre-incubation of sperm in a capacitating medium affected all the parameters studied (Table 1B). Penetration increased proportionally to the concentration of $\text{HCO}_3^-$ in sperm pre-incubation, although monospermy was more closely related to the $\text{HCO}_3^-$ concentration in the IVF medium, reaching between 21.5% and 25.4% when IVF was carried out at 25 mmol/L and between 36.2% and 48.7% when it was carried out at 15 mmol/L. Thus, the higher efficiency was achieved when both pre-incubation and IVF was performed in 15 mmol/L (33.9 ± 3.7%, $P < 0.05$).

**Discussion**
Spermatozoa maturation in the epididymis comprises sequential modifications that have been demonstrated to be essential for the acquisition of motility and fertility [37]. Once in the epididymal cauda, where the $\text{HCO}_3^-$ concentration is minimal (~ 4 mmol/L), matured spermatozoa remain in a quiescent state. After ejaculation, spermatozoa are activated and changes in the sperm membrane begin as they progress through the uterus, where they find increasing concentrations of $\text{HCO}_3^-$. Subsequently, sperm capacitation is completed in the
female isthmus and spermatozoa are again progressively exposed to increasing amounts of the capacitating effector $\text{HCO}_3^-$ from the isthmus to ampulla [38]. In the present study we have elucidated how epididymal spermatozoa respond to different $\text{HCO}_3^-$ concentrations during capacitation and in vitro fertilisation. This study was carried out using mature spermatozoa that had never been in contact with high concentrations of $\text{HCO}_3^-$ or with decapacitant agents from accessory sex glands. Epididymal spermatozoa were incubated in a capacitating medium containing 0, 5, 15 or 25 mmol/L of $\text{HCO}_3^-$ and in non-capacitating medium. The results showed that the highest degree of PKAs activation was achieved by incubation in a capacitating medium containing 15 mmol/L of $\text{HCO}_3^-$ or above, although the 25 mmol/L concentration did not perform any better than 15 mmol/L. In spite of the presence of other capacitating agents such as calcium and albumin, concentrations lower than 15 mmol/L (for example the 5 mmol/L, which is similar to that existing in the epididymis) produced a low pPKAs, similar to that produced in the absence of $\text{HCO}_3^-$ (0 mmol/L), quantitatively matching the NCAP conditions which lack other capacitation effectors as albumin and calcium. This fact reflects that not only $\text{HCO}_3^-$ is necessary to initiate the capacitation cascade but also albumin and calcium, as has been previously described. It is known that defective $\text{HCO}_3^-$ secretion into the uterus may be related to impaired sperm capacitation and a low female fertility [9]. Therefore, a 15 mmol/L concentration of $\text{HCO}_3^-$ is postulated as being sufficient to achieve a high degree of PKAs activation that is essential in the sperm capacitation process. The differences in the results obtained with different $\text{HCO}_3^-$ concentrations were not dependent on the viability of the cells analysed (Additional file 4). Therefore, although this study showed the dose-dependent effect of $\text{HCO}_3^-$ on sperm capacitation, above 15 mmol/L there was no extra effect. However, the kinetic of PKAs activation allowed us to understand that the

Fig. 5 Comparison of epididymal sperm trajectories in capacitating conditions using different $\text{HCO}_3^-$ concentrations. Boar epididymal spermatozoa were incubated in capacitating medium (TALP) containing different concentrations of $\text{HCO}_3^-$ (0 mmol/L, 5 mmol/L, 15 mmol/L and 25 mmol/L) for 60 min. Sequences of 2 s were obtained by CASA (50 frames/s).
required time to activate PKAs does depend on HCO$_3^-$ concentration, which was rapidly activated at 1 min of incubation at concentrations of 15 and 25 mmol/L. These results agree with a previous study [6]. Furthermore, PKAs was highly phosphorylated after 30 min of incubation with 25 mmol/L and after 60 min with 15 mmol/L, demonstrating that a concentration of 15 mmol/L can produce a high degree of pPKAs, although it takes longer. The absence (0 mmol/L) or low concentrations of HCO$_3^-$ (5 mmol/L), similar to that existing in the cauda epididymis, did not enable PKAs activation even after 60 min of incubation, which shows that not only is the presence of HCO$_3^-$ essential in this pathway but also a minimum concentration is required. HCO$_3^-$ is a key activator of a cascade of events in capacitation and a minimum concentration and incubation time is necessary to trigger the capacitation process and produce a high pPKAs, despite the presence of other capacitating agents like calcium and albumin. 15 mmol/L of HCO$_3^-$ seems to be a sufficient concentration to allow epididymal sperm capacitation. Nevertheless, the importance of HCO$_3^-$ not only lies in the activation of PKAs but also the fact that it produces another series of events essential for sperm physiology. The increase in intracellular pH conditions, a phenomenon during capacitation (reviewed by Nishigaki et al. [39]) and the Adcy10/cAMP/PKA pathway are not an exception.

Epididymal spermatozoa in the cauda epididymis remain in a quiescent state favoured by low HCO$_3^-$ content and acidic pH [10]. The present study showed that, in the absence of HCO$_3^-$ and the presence of other capacitating agents like calcium and albumin, the pH remained at 6.7–6.8. In the presence of HCO$_3^-$, the pH increased 0.3–0.4 units, confirming that the increase depends exclusively on HCO$_3^-$ This increase of around 0.3–0.4 units in pH may seem small, but, it has been demonstrated that an increase of 0.2 units of pH may modify enzymatic activity more than 20-fold [40]. It is known that the activity of intra- and extra-cellular enzymes taking part in the cellular metabolism is pH-sensitive.

The effect of HCO$_3^-$ has been widely studied and there is evidence that extracellular HCO$_3^-$ influences the motility of epididymal spermatozoa of several species [10, 41, 42]. In the present study, HCO$_3^-$ concentration did not affect the proportion of motile spermatozoa during incubation in a capacitating medium, or their progressivity, but did affect parameters related to sperm trajectory. Holt and Harrison [43] showed in ejaculated, preserved and selected spermatozoa by Percoll gradient that the straight and average velocities, straightness and linearity significantly increased with the addition of HCO$_3^-$ to the incubation medium. The results of the present study presented evidence that HCO$_3^-$ activates motility in epididymal spermatozoa and produces a change in their trajectory which becomes more linear (see Fig. 5) and rapid. These data suggest that HCO$_3^-$ is not only important for motility activation in a balanced salt solution but also that the type of movement produced is concentration-dependent. A concentration of
15 mmol/L HCO₃⁻ during sperm capacitation produced a more linear movement, which has previously been related with the state of capacitation in boar [43, 44].

The HCO₃⁻ induction of an increase in LIN in porcine is controversial, since it has been previously reported by Satake et al. [45] using 15 mmol/L HCO₃⁻, but it is in clear contrast to other studies [46] in which hyperactivation of ejaculated preserved spermatozoa was related to a decreased LIN. However, sperm motility in capacitating conditions seems to be species-specific, for instance in rodents as rat and hamster the hyperactivated motility is characterised by a decreased LIN [47, 48]. In our study, HCO₃⁻ concentrations lower than 15 mmol/L produced a more curvilinear trajectory, while higher concentrations (25 mmol/L) did not produce any extra improvement over the value obtained at 15 mmol/L. However, each individual spermatozoon responds differently to HCO₃⁻ [45] and the clustering analysis identified 3 different sperm subpopulations according to their trajectory in response to HCO₃⁻. In this case, the proportion of spermatozoa with more linear trajectory was higher in the presence of HCO₃⁻. While a concentration of HCO₃⁻ from 15 mmol/L stimulated sperm motility and produced a change in the type of trajectory, no additional effect was obtained with 25 mmol/L. The role of HCO₃⁻ in sperm capacitation is not only restricted to the immediate effects on spermatozoa (increased pH, activation of motility and pPKAs) but is also important in slow capacitation events such as the increase in Tyr-P and AR. In vitro sperm capacitation is accompanied by a time-dependent increase in the Tyr-P of a subset of proteins, as one of the final steps of the PKA-dependent cascade of capacitation [5]. Sperm incubated in a medium lacking HCO₃⁻ fails to undergo capacitation, but the process can be recovered by adding HCO₃⁻ in a concentration-dependent way [5]. The present study showed that a concentration of 15 mmol/L is sufficient to produce an intracellular alkalinisation and a high
Table 1  Effect of HCO$_3^-$ concentration on in vitro sperm fertility. A) Monophasic IVF: performed in TALP medium containing different HCO$_3^-$ concentrations (0 mmol/L, 5 mmol/L, 15 mmol/L and 25 mmol/L) for 18 h of co-culture at 38.5 °C. B) Biphasic IVF (Sperm pre-incubation + Monophasic IVF): sperm were pre-incubated in TALP for 60 min in 5 mmol/L, 15 mmol/L or 25 mmol/L and then IVF was performed in TALP medium with the same or higher HCO$_3^-$ concentration for 18 h of co-culture at 38.5 °C

A

| [HCO$_3^-$], mmol/L | n  | Pen, % | Mon, % | SPZ/O | SPZ/ZP | Efficiency, % |
|---------------------|----|--------|--------|-------|--------|---------------|
| 0                   | 216| 0$^a$  | –      | –     | 0.3 ± 0.1$^a$ | 0$^a$          |
| 5                   | 204| 21.6 ± 2.9$^b$ | 61.4 ± 7.4$^a$ | 1.7 ± 0.2$^a$ | 28 ± 0.2$^b$ | 13.2 ± 2.4$^b$ |
| 15                  | 206| 79.6 ± 2.8$^b$ | 32.9 ± 3.7$^b$ | 4.0 ± 0.3$^b$ | 49 ± 0.5$^b$ | 26.2 ± 3.1$^c$ |
| 25                  | 280| 97.5 ± 0.1$^d$ | 8.8 ± 1.7$^c$ | 6.6 ± 0.3$^c$ | 8.8 ± 0.5$^d$ | 8.6 ± 1.7$^d$ |

B

| [HCO$_3^-$], mmol/L | n  | Pen, % | Mon, % | SPZ/O | SPZ/ZP | Efficiency, % |
|---------------------|----|--------|--------|-------|--------|---------------|
| Sperm pre-incubation |    |        |        |       |        |               |
| IVF                 | 5  | 15     | 171    | 46.8 ± 3.8$^a$ | 36.2 ± 5.4$^{ab}$ | 3.0 ± 0.2$^{ab}$ | 3.4 ± 0.2$^a$ | 17.0 ± 2.9$^a$ | 17.0 ± 2.9$^a$ |
| 5                   | 5  | 15     | 172    | 54.1 ± 3.8$^a$ | 21.5 ± 4.3$^a$ | 4.4 ± 0.3$^a$ | 4.1 ± 0.3$^{bc}$ | 4.1 ± 0.3$^{bc}$ | 11.6 ± 2.4$^a$ |
| 15                  | 15 | 165    | 76.8 ± 3.3$^{bc}$ | 25.4 ± 3.9$^a$ | 3.6 ± 0.2$^{bc}$ | 4.5 ± 0.3$^{bc}$ | 19.5 ± 3.1$^a$ |
| 25                  | 25 | 171    | 86.6 ± 2.6$^b$ | 24.3 ± 3.5$^b$ | 3.4 ± 0.2$^b$ | 4.8 ± 0.3$^{bc}$ | 21.0 ± 3.1$^a$ |

Results are expressed as mean ± SEM. Different superscripts ($^a$–$^d$) in the same column indicate significant differences (P < 0.05).

Pen, % penetration percentage of total oocytes used. Mon, % monospermy percentage calculated from penetrated oocytes. SPZ/O number of spermatozoa penetrating each oocyte. SPZ/ZP number of sperm bound to ZP. Efficiency, % final number of putative zygotes (monospermic) in each group per 100 penetrated oocyte.

degree of Tyr-P in spermatozoa but no extra effect was observed when 25 mmol/L was used. Something similar occurred with Tyr-P in the flagellum of spermatozoa. A high degree of pattern IV was observed at 15 mmol/L and above, although no significantly improvement was observed in 25 mmol/L. Overall, this study agrees with those of other authors [49] who showed that HCO$_3^-$ is required to activate Adcy10, which increases intracellular cAMP, promotes PKA activity and subsequent Tyr-P. The above-mentioned observations concerning the role of HCO$_3^-$ concentration on sperm functionality have shown the modulating power of HCO$_3^-$ on sperm physiology in capacitating conditions. However, whether this modulating effect influences the sperm fertilising ability during IVF has not been elucidated. In this study, the effect of HCO$_3^-$ concentration on sperm fertility was investigated (Table 1A). We hypothesised that varying the HCO$_3^-$ concentration in the porcine sperm capacitation process regulate their functionality and probably affect IVF output. Indeed, IVF was directly and strongly affected by the HCO$_3^-$ concentration in the medium. The importance of HCO$_3^-$ in the fertilisation process was confirmed by the fact that no oocyte was penetrated in the absence of HCO$_3^-$ (0 mmol/L). This agrees with Suzuki et al. [50], who described how the absence of HCO$_3^-$ in fertilisation media makes oocyte fertilisation impossible. In our study, we found that HCO$_3^-$ exerts a concentration-dependent impact on fertilisation as penetration increased with increasing HCO$_3^-$ concentrations. In addition, the presence of HCO$_3^-$ also upregulated the SPZ/O resulting in monospermy reduction.

The high incidence of polyspermy in porcine has been widely related with an unconacceptably low efficiency of IVF [51]. Hunter and Nichol [52] hypothesised that the incidence of polyspermy is a direct consequence of the number of capacitated spermatozoa in the immediate vicinity of ovulated porcine oocytes in vivo. Later, Abydeera and Day [21] also described this correlation under in vitro conditions. Results from this manuscript suggest that the effect on IVF of a specific HCO$_3^-$ concentration is related with the timing of the capacitation events and their influence in monospermic fertilisation success. At the molecular level, our results indicate that 5 mmol/L did not activate PKAs while 25 mmol/L produce faster phosphorylation compared to 15 mmol/L. The slower PKAs activation of 15 mmol/L could be responsible for the lower penetration and the higher monospermy than observed with 25 mmol/L in the fertilisation experiments. Moreover, Funahashi [53] described that one of the strategies to reduce polyspermy in porcine is to induce full AR in partially reacted spermatozoa. However, the results of the AR assay (Additional file 5) showed that polyspermy is not related with the proportion of acrosome-reacted spermatozoa surrounding the egg. Concentrations of 15 and 25 mmol/L of HCO$_3^-$ in the capacitating media produce the same proportion of acrosome-reacted sperm after 60 min of incubation.

In addition to its effects in sperm penetration and monospermy parameters, HCO$_3^-$ had a determining action in the
primary recognition between gametes, since practically no spermatozoon was able to bind to ZP in the absence of HCO$_3$\textsuperscript{−}. As Flesch et al. [54] suggested, it is probable that the absence of HCO$_3$\textsuperscript{−} hindered certain capacitation events that resulted in reduced binding affinity of spermatozoa for ZP. One important question is the extent by which the influence of HCO$_3$\textsuperscript{−} in IVF is exerted on spermatozoa during capacitation or on gamete co-incubation during the fertilisation process. After sperm pre-incubation with 15 mM/L, which produced a moderate penetration, when IVF was also carried out at 15 mM/L, monospermy was significantly higher and the SPZ/O lower than in the rest of groups, which resulted in a higher final efficiency (33.9%). What it is clear is that oocyte penetration directly increased with the HCO$_3$\textsuperscript{−} concentration in the sperm pre-incubation medium suggesting that the action of HCO$_3$\textsuperscript{−} is exerted more on spermatozoa than on oocytes. Very few studies have described the effect of HCO$_3$\textsuperscript{−} on in vitro matured oocytes, but Suzuki et al. [50] suggested that, although HCO$_3$\textsuperscript{−} was apparently affecting gamete interaction, it is highly probable that HCO$_3$\textsuperscript{−} affects fertilisation through its effect on spermatozoa rather than through any effect on the oocytes. 

Conclusion

Altogether our results suggest that 15 mM/L HCO$_3$\textsuperscript{−} is sufficient for IVF media because it produces a high degree of sperm capacitation and maximises IVF efficiency in porcine. In conclusion, adjusting the HCO$_3$\textsuperscript{−} concentration of the medium during in vitro epididymal sperm capacitation and fertilisation allowed us to increase the monospermy efficiency of these in vitro processes in porcine, which are currently suboptimal. In addition, a concentration of 15 mM/L HCO$_3$\textsuperscript{−} in the capacitating medium is also sufficient to activate the sperm capacitation cascade and induce the slower progression of the sAC/cAMP/PKA pathway. A concentration of 15 mM/L produces a sufficient level of activation of sperm motility, pH$_i$ increase and Tyr-P, maintaining high levels of fertilizing ability and increased rates of monospermy.

Additional files

**Additional file 1:** Effect of HCO$_3$\textsuperscript{−} on boar epididymal sperm intracellular pH (pH$_i$). A Calibration of the system in which fluorescence emission ratio 490/440 nm was detected when stained spermatozoa with BCECF-AM were exposed to different extracellular pH (pH$_e$) in the presence of 0.1% Triton X-100 and excited both at 490 and 440 nm. The regression line for pH$_e$ vs. the 490/440 nm ratio was obtained (y = 3.048x + 15.534) and pH$_i$ of sperm cells was estimated from that regression line. B Detection of fluorescence (arbitrary units) for 300 s when BCECF-AM stained spermatozoa were excited both at 490 (dark grey line) and 440 nm (light grey line) and the ratio 490/440 nm (black line). (TIF 7 kb)

**Additional file 2:** Cluster centers for each CASA motility parameter used to classify each spermatozoa of the study according to their degree of similarity. Cluster 1: spermatozoa with the poorest motility (low values in all CASA parameters); Cluster 2: spermatozoa with the most linear trajectory (high values of VSL, LIN, STR and WOB); Cluster 3: spermatozoa with the most curvilinear trajectory (high values of VCL and ALH and low values of VSL, LIN and STR); VCL, μm/s: curvilinear velocity; VSL, μm/s: straight-line velocity; VAP, μm/s: average path velocity; LIN, %: linearity of the curvilinear trajectory; STR, %: straightness; WOB, %: Wobble (VAP/VCL); ALH, μm: amplitude of lateral head displacement; BCF, Hz: beat cross-frequency. (PDF 28 kb)

**Additional file 3:** Patterns of immunolocalization of protein tyrosine phosphorylation (Tyr-P) on boar epididymal sperm. The Tyr-P location of spermatozoa proteins were classified and grouped into 4 different categories according to the four patterns described by Luho et al. [28]: I Pattern I (low capacitation status), which included spermatozoa without fluorescence (A) or with phosphorylated acrosome (B) or tail (C) or acrosome and tail (D); II Pattern II (medium capacitation status), which included spermatozoa with fluorescence in the equatorial subsegment with (E) or without (F) the presence of signal in the flagellum; iii) Pattern III (high capacitation status), which included spermatozoa with signal in the equatorial subsegment and acrosome area and with (G) or without (H) the presence of signal in the flagellum; Pattern IV (motility activation status), which included those spermatozoa with a signal in the flagellum regardless of other locations (I). (TIF 12 kb)

**Additional file 4:** A) SpERM plasma membrane integrity evaluated with eosin-nigrosin staining as a reflection of spermatozoa viability. Spermatozoa viability was classified as i) viable: membrane-intact spermatozoa, impermeable to eosin-nigrosin staining (black arrow) or ii) non-viable: membrane-altered spermatozoa, permeable to eosin-nigrosin staining (black asterisk). B) Effect of HCO$_3$\textsuperscript{−} concentration on sperm viability. Epididymal sperm samples were incubated at 38.5 °C for 1 and 60 min in a capacitating medium (TALP) with different concentrations of HCO$_3$\textsuperscript{−} (0 mM/L, 5 mM/L, 15 mM/L and 25 mM/L) and in non-capacitating medium (NCP). Sperm plasma membrane integrity was evaluated using the eosin-nigrosin staining technique. Seven replicates were performed, in which 200 spermatozoa per experimental group were classified as viable (membrane-intact) or non-viable (membrane-altered). Results are shown as mean ± SEM. Data were analysed by one-way ANOVA and a Tukey test of multiple comparisons. Different letters (a-b) indicate statistically significant differences between groups in the same time of incubation (P < 0.05). (TIF 9 kb)

**Additional file 5:** Effect of HCO$_3$\textsuperscript{−} concentration on acrosome reaction (AR). Epididymal sperm samples were incubated at 38.5 °C for 1 and 60 min in a capacitating medium (TALP) with different concentrations of HCO$_3$\textsuperscript{−} (0 mM/L, 5 mM/L, 15 mM/L and 25 mM/L) and in non-capacitating medium (NCP). AR was assessed by staining with 1% FITC-conjugated peanut agglutinin from Arachis hypogaea (PNA-FITC L7381, Sigma-Aldrich®, Madrid, Spain) 20 μg/mL. Samples were fixed at 1% formal-saline, smeared onto glass slides and coverslips and analysed by epifluorescence microscopy (blue filter, BP 480/40; emission BP 527/30; Leica® DM4000 B LED, USA) at x 400. Four replicates were performed, in which 200 spermatozoa per sample were analysed, classifying the sperm in reacted or non-reacted (with or without fluorescence in the acrosomal region, respectively). Results are shown as mean ± SEM. Data were analysed by one-way ANOVA and a Tukey test of multiple comparisons. Different letters (a-b) in the same time of incubation indicate statistically significant differences (P < 0.05). (TIF 4 kb)

**Abbreviations**

Adcy10: Atypical soluble adenylyl cyclase Adcy10 aka sAC; ALH: Amplitude of lateral head displacement; AR: Acrosome reaction; BCF: Beat cross-frequency; BSA: Bovine serum albumin; CASA: Computer-assisted spermatozoa motility analysis; COCs: Cumulus-oocytes complexes; HCO$_3$\textsuperscript{−}: Bicarbonate; IIF: Indirect immunofluorescence; IVF: In vitro fertilisation; LIN: Linearity of the curvilinear trajectory; Mon: Percentage of monospermy of penetrated oocytes; Mot: Percentage of total motile spermatozoa; MotPro: Motile progressive spermatozoa; NBC: Electrogenic Na$^+$/HCO$_3$\textsuperscript{−} co-transporter; NCP: Non-capacitating control; PBS: Phosphate buffer saline; Pen: Percentage of penetrated oocytes; pH$_f$: Extracellular pH; pH$_i$: Intracellular pH; PKA: Protein kinase A; pPKAs: Phosphorylation of protein kinase A substrates; STR: Straightness; TALP: Tyrode’s albuminate lactate pyruvate media; Tyr-P: Tyrosine-phosphorylated spermatozoa; WOB, %: Wobble (VAP/VCL); ZP: Zona pellucida.
P: Tyrosine phosphorylation; VAP: Average path velocity; VCL: Curvilinear velocity; VSL: Straight line velocity; WOB: Wobble of the curvilinear trajectory; ZP: Zona pellucida; β-act: Anti-β-actin HRP; β-tub: Anti-β-tubulin

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Availability of data and materials
All data generated or analysed during this study are included in this published article and its supplementary information files.

Authors’ contributions
CS-U performed the experiments, collected the data and conducted the laboratory procedures and statistical analyses and wrote the manuscript. JR-A performed the motility experiment and analysed the data. CM contributed to the manuscript preparation. FAG-V and PE-V conceived the experiments. FAG-V designed and directed the experiments and contributed to the manuscript preparation. All the authors have revised the final version of the manuscript.

Ethics approval
The study was carried out following the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63/UE on animal protection. All the procedures carried out in this work were approved by the Ethical Committee of Animal Experimentation of the University of Murcia and by the Animal Production Service of the Agriculture Department of the Region of Murcia (Spain) (ref. no. A13160609).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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