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SHORT COMMUNICATION

Boar sperm motility is modulated by CCK at a low concentration of bicarbonate under capacitation conditions

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Abstract

In a previous study, our group detected the cholecystokinin (CCK) protein in the porcine oviduct. This fact, together with the involvement of CCK in the regulation of sperm protein tyrosine phosphorylation by the modulation of HCO_3^- uptake (in mice and humans) suggests a role for CCK during sperm capacitation. Therefore, on the one hand, the expression of CCK receptors (CCK1R and CCK2R) on boar testes has been investigated and probed; on the other hand, boar spermatozoa (from seminal doses of 1-day and 5-day storage) were exposed to different concentrations of CCK (0-control, 25 or 50 μM) in a medium supporting capacitation supplemented with 0, 5 or 25 mmol/L of HCO_3^- for 1 h at 38.5°C. Sperm motion (total and progressive motility), kinetic parameters, viability, acrosome status, and mitochondrial activity were determined. No differences between groups (0, 25 or 50 μM of CCK) were observed when HCO_3^- was absent in the media ($p > .05$). However, the results showed that when the media was supplemented with 5 mmol/L HCO_3^- in 1-day seminal dose storage, the linearity index (LIN, %), straightness index (STR, %) and oscillation index (WOB, %) (sperm kinetics parameters) increased in the presence of CCK regardless the concentration ($p < .05$). Nevertheless, CCK in sperm from 5-day storage only increased the WOB parameter in comparison to the control ($p < .05$). Furthermore, the average amplitude of the lateral displacement of the sperm head (ALH, μm) and curvilinear velocity (VCL, μm/s) decreased when CCK was present, depending on its concentration and sperm aging (1-day vs. 5-days) ($p < .05$). In the case of the media supporting capacitation supplemented with 25 mmol/L HCO_3^-, any differences were observed except for sperm viability in the 5-day seminal doses, which increased in the 50 μM-CCK group compared to the control ($p < .05$). In conclusion, these data suggest an implication of CCK protein during sperm capacitation under low bicarbonate concentration increasing the sperm linear trajectory.

KEYWORDS
bicarbonate, CASA, fertilization, pig, sperm function, Spermatology

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1 | INTRODUCTION

The oviduct is a tubular organ that plays an important role in reproductive events. It provides the appropriate microenvironment for gamete transportation and maturation, sperm capacitation, fertilization, and early embryo development (Coy et al., 2012; González-Brusi et al., 2020; Hunter, 1998; Pérez-Cerezales et al., 2018). The oviductal fluid (OF) is produced by the secretion of the oviductal epithelial cells and from a transudate of blood plasma and is composed of metabolites, amino acids, inorganic salts, lipids, glycosaminoglycans, proteins, and extracellular vesicles (Almiñana & Bauersachs, 2019; Aviles et al., 2010; Leese et al., 2001; Leese et al., 2008). It has been demonstrated that the components synthesized and secreted by the oviduct interact with and influence the activities of gametes and the development of early embryos (Aviles et al., 2010; Coy et al., 2012; González-Brusi et al., 2020; Maillo et al., 2016; Suarez, 2007; Suarez, 2008a, 2008b; Talevi & Gualtieri, 2010).

Cholecystokinin (CCK) is a gastrointestinal hormone discovered in 1966 as a peptide extracted from porcine intestine (Jorpes & Mutt, 1966). In a previous study, our group analysed the gene expression in the porcine oviduct during the oestrous cycle, and for the first time, the protein CCK was detected in this organ (Acuña et al., 2017). Its expression in the oviduct was confirmed by RT-qPCR throughout the oestrous cycle, being upregulated in the postovulatory stage compared to the preovulatory and luteal stages. Furthermore, it was also detected in the epithelial cells in all phases of the oestrous cycle by immunohistochemical analysis and in the OF by proteomic analysis (Acuña et al., 2017). Its presence in the OF in the postovulatory stage could indicate a role in sperm functionality. Thus, CCK expression was found in rat epididymis (Persson et al., 1989) and guinea pig, monkey, mouse, pig, rat, and chicken testis (Persson et al., 1988; Persson et al., 1989; Wan et al., 2023). On the other hand, CCK-like peptides were detected in spermatocytes and spermatids of monkey, mouse, rat, and human species (Pelto-Huikko et al., 1989; Persson et al., 1989; Schalling et al., 1990); specifically in the sperm acrosome of monkey and human (Pelto-Huikko et al., 1989; Persson et al., 1989; Schalling et al., 1990); and CCK and its receptors (CCK1R and CCK2R) have also been detected in the acrosome region of mature mouse sperm (Zhou et al., 2015). The involvement of CCK and its receptors in the regulation of protein tyrosine phosphorylation in a dose-dependent manner by HCO_3^- uptake modulation has been demonstrated in human and mouse sperm (Zhou et al., 2015). CCK peptides located in the sperm acrosome have been suggested to be released during the acrosome reaction, which might facilitate sperm motility during egg penetration (Pelto-Huikko et al., 1989; Persson et al., 1988, 1989; Sasse et al., 2000). CCK and its receptors were also detected in chicken's ovary and uterus (Wan et al., 2023), and CCK receptors on frog oocytes (Moriarty et al., 1988), although, as far as we are concerned, no receptors have been described in mammalian oocytes or mammalian female reproductive tissues.

At present, the literature has identified the CCK as a protein involved in sperm capacitation by the modulating tyrosine phosphorylation in some species (Zhou et al., 2015), but any information regarding the impact of this protein on sperm function has been published in porcine species. In the worldwide animal husbandry industry, pigs are a primary source of meat protein (Ritchie et al., 2017), but the use of in vitro production embryos for a faster genetic spread is limited because of polyspermy (Romar et al., 2019). Thereby, increasing knowledge of sperm behaviour during capacitation may help with assisted reproductive techniques (ARTs) like artificial insemination and in vitro fertilization. Therefore, this work aimed to study the expression of CCK receptors on pig testes and examine the effect of CCK protein (0, 25, and 50 μM) on sperm function (motion parameters, viability, acrosome status, and mitochondrial activity) under capacitation conditions (media supporting capacitation with 5 or 25 mmol/L HCO_3^-) (Soriano-Úbeda et al., 2019).

2 | METHODS

2.1 | Reagents

The reagents used in the study were, unless otherwise indicated, acquired from Sigma-Aldrich®.

2.2 | Purification of testis RNA, cDNA synthesis and PCR amplification of *CCK1R* and *CCK2R* cDNA

Testes (Landrace x Large White, $n=3$) were collected from a local slaughterhouse (El Pozo Alimentación S.A., Alhama de Murcia, Murcia, Spain) within 15–20 min of death. Total RNA was isolated using the RNAqueous kit (Ambion, Austin, TX, USA), following the manufacturer's instructions. The first strand of cDNA was synthesized from total RNA with the QuantiTect Reverse Transcription Kit using reverse-transcribed polymerase chain reaction (PCR; Qiagen, Venlo, Netherlands), according to the manufacturer's protocol. To amplify CCK1 and CCK2 receptors, specific primers were designed with the NCBI primer tool according to the predicted cDNA sequences in the GenBank database with accession numbers XM_013978565 and XM_021062350 for CCK1R and CCK2R, respectively. B-actin (ACTB; Accession no. XM_021086047) was used as a positive control (Table 1). PCR was performed using the KAPA2G Fast HotStart ReadyMix (Roche, Basel, Switzerland), with an initial denaturation cycle of 3 min at 95°C, followed by 35 cycles of 15 s at 95°C, 15 s at 55°C, and then 1 s at 72°C. The final extension time was 1 min at 72°C. The generated amplicons were visualized by 1.5% agarose gel electrophoresis and purified using the FavorPrep™ GEL/PCR Purification Kit (FAVORGEN Biotech Corp., Vienna, Austria) to be sequenced by Sanger using a 3500 GeneticAnalyzer (Applied Biosystems).

2.3 | Sperm collection and preparation

The sperm-rich fraction of ejaculates from Duroc boars (*Sus scrofa*) with proven fertility was used for the experiments in this

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TABLE 1 Primers used for PCR analysis of the CCK1 and CCK2 receptors, designed using the NCBI primer tool.

Gene name	GenBank accession number	Primer orientation	Primer sequence (5′-3′)
CCK1R	XM_013978565	Forward	CGG GCT TTC TTC TGC TTG TG
		Reverse	GCA GAG TCT CCC AAG TGC AA
		Forward	CCC ATG CAT GTG TCT GTC CA
		Reverse	GCA TGT GCA CTC CTG TCT TG
CCK2R	XM_021062350	Forward	TTC TGA TGA GTG TCG CCG GA
		Reverse	AGT AGG AAA CCG CCT TGC AC
ACTB	XM_021086047	Forward	GTC ACC AAC TGG GAC GAC AT
		Reverse	GGC AGC TCG TAG CTC TTC TC

study. A total of 7 ejaculates from different boars were collected by masturbation (gloved hand technique) in a pre-warmed thermal cup. Semen samples ($n = 7$, once per ejaculate) were diluted in AndroStar® Plus extender (Minitüb, Tiefenbach, Germany) until they reached a final concentration of ~33×10^6 sperm/ml. Semen was packaged in plastic bags (2000×10^6 sperm/60 mL) and transported to the laboratory within 12 h from the extraction at 16°C, protected from light. The inclusion criteria established for using the ejaculates were: total motility >70%, viable sperm >90%, and morphoanomalies ≤10%.

After seminal dose conservation for 1 or 5 days (16°C) (day 1 was considered 24 h after the preparation of the seminal dose), samples were centrifuged (500***g***, 10 min; centrifuge Model 5418 R, Eppendorf®, Hamburg, Germany), discarded the supernatant, and resuspended in modified Tyrode's albumin lactate pyruvate media (TALP), based on the description by Rath et al. (Rath et al., 1999), until reaching a final concentration of ~33×10^6 sperm/ml. Prior to sperm incubation, TALP media was supplemented with 0, 5 or 25 mmol/L HCO_3^- and all media were adjusted to pH 7.4. The pH of the medium containing 0 mmol/L of HCO_3^- was balanced using NaOH and HCl, and warmed in a heat block at 38.5°C (Accu Block®, Labnet International, Inc., New York, USA). The pH of the media containing 5 and 25 mmol/L of HCO_3^- was adjusted in an incubator for nearly 3 h at 38.5°C, saturated humidity, and 1% or 5% of CO_2 atmosphere in the air, respectively, according to the Henderson-Hasselbalch equation.

Once the pH was adjusted, the TALP media was supplemented with different CCK (CCK-8 Cholecystokinin Octapeptide sulphated, Bachem, Bubendorf, Switzerland) concentrations (0 μM-control, 25 μM or 50 μM). Then, the spermatozoa samples were incubated at 38.5°C for 1 h in the different TALP media conditions (0, 5 or 25 mmol/L HCO_3^-) supplemented with CCK (0, 25 or 50 μM) in the same conditions previously described for the equilibration of the media.

2.4 | Sperm motility analysis

A sample of 4 μL was placed in a warmed (38.5°C) chamber (SpermTrack20®, PROiSER R+DSL, Valencia, Spain) and a 10× objective negative-phase in a phase-contrast microscope (Leica® DMR, Wetzlar, Germany) and digital camera (Basler Vision, Ahrensburg, Germany) were used to perform the evaluations. Motility determinations were made at 25 frames per second for 1 s (25 images), and sperm were considered to be motile and progressive when VAP >10 μm/s and STR >45%, respectively. The motility parameters, determined using the ISAS® Software System (PROiSER R+DSL, Valencia, Spain), were: total and progressive motility (%), curvilinear velocity (VCL, μm/s), rectilinear velocity (VSL, μm/s), average speed (VAP, μm/s), linearity index (LIN, %), straightness index (STR, %), oscillation index (WOB, %), average amplitude of the lateral displacement of the head (ALH, μm), and beat-cross frequency (BCF, Hz).

2.5 | Viability analysis

The sperm viability was evaluated with propidium iodide (PI). Sperm samples were incubated with the staining solution (50 μL of PI [500 μg/mL]) in 10 mL PBS (Phosphate buffer solution) without Ca^{2+} and Mg^{2+} for 10 min at room temperature under darkness. Then, sperm viability (at least 200 sperm cells per sample) was evaluated under a fluorescence microscope (Leica® DM4000 Led, Wetzlar, Germany, 495/520 nm) and classified as live (without fluorescence) and dead (red fluorescence).

2.6 | Acrosome status analysis

The sperm acrosome status was evaluated with the *Arachis hypogaea* lectin (PNA-FITC). Sperm samples were incubated with PNA-FITC solution (100 μL of PNA-FITC [200 μg/mL] in 10 mL of PBS without Ca^{2+} and Mg^{2+}) for 10 min at room temperature under darkness. Then, sperm acrosome status (at least 200 sperm cells per sample) was evaluated under a fluorescence microscope and classified as sperm with a normal apical ridge (without fluorescence) and a damaged apical ridge (green fluorescence).

2.7 | Mitochondrial activity analysis

The sperm mitochondrial activity was evaluated with JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide;

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Thermo Fisher Scientific Inc., MA, USA). Sperm samples were incubated with JC-1 solution (10 μL of JC-1 [0.017 μg/mL] in 10 mL of PBS without Ca^{2+} and Mg^{2+}) for 30 min at 38.5°C under darkness. Then, sperm samples (at least 200 sperm cells per sample) were evaluated under fluorescence microscopy and classified as sperm with low and high mitochondrial membrane potential (green and orange fluorescence, respectively).

2.8 | Statistical analysis

Statistical analyses were performed with the SPSS 24.0 software package (IBM SPSS Inc., Chicago, IL, USA). Sperm quality parameters ($n=7$ replicates) were analysed for normality by the Shapiro–Wilk test. A one-way ANOVA test followed by a post-hoc Tukey test was applied. For variables whose data were not normally distributed, the non-parametric Kruskal–Wallis test was used. Data are represented as the mean ± SEM (standard error of the mean). Differences were considered significant when $p<.05$.

3 | RESULTS

3.1 | CCK receptors are expressed in the testes

CCK1R and CCK2R amplicons with the expected molecular weight (397 and 204 bp, respectively) were detected in boar testes (Figure 1). Sequencing demonstrated an identity of 100% with the cDNA sequences in the GenBank database with accession numbers XM_013978565 and XM_021062350 for CCK1R and CCK2R, respectively.

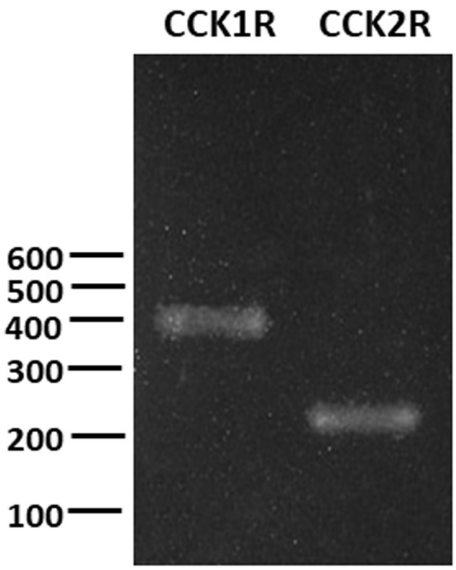


FIGURE 1 Analysis of CCK1R and CCK2R gene expression in pig testis as determined by reverse transcription–polymerase chain reaction (RT-PCR), 397 and 204 bp amplicons for the CCK1R and CCK2R genes, respectively, are shown.

3.2 | Exogenous CCK modulates sperm kinetics under capacitation conditions

When the medium supporting capacitation was devoid of HCO_3^- (0 mmol/L), the presence of CCK had no effect in any sperm parameter analysed ($p>.05$), neither regarding the CCK concentration (0, 25, or 50 μM) nor the sperm aging (1-day vs. 5-day semen storage). In the same sense, the highest concentration of HCO_3^- (25 mmol/L HCO_3^-) in the medium had no effect over the sperm motility analysis (Figure 2), and only provided significantly higher sperm viability (5-day storage) in the 50 μM CCK group compared to the control (70.42 ± 0.65% vs. 67.28 ± 0.60%, $p=.021$), but without statistical differences regarding the 25 μM CCK group (68.71 ± 0.60%, $p>.05$). Interestingly, the presence of CCK affected most of the sperm kinetic parameters when the medium was supplemented with 5 mmol/L HCO_3^- (Figure 3). In this sense, CCK increased LIN, STR and WOB sperm parameters in 1-day doses (for both, 25 and 50 μM; $p<.05$) compared to the control, but only WOB parameter increased in 5-day doses, when 50 μM CCK was added, in comparison to the control ($p<.05$) (Figure 3g–i). Moreover, the groups containing CCK (25 or 50 μM) decreased VCL and ALH parameters comparing to the control, but differently, depending on sperm aging and CCK concentration (Figure 3c,f). Then, the presence of CCK decreased VCL and ALH in the sperm of both types of seminal doses (1- or 5-day storage), although only the highest CCK concentration (50 μM, but not 25 μM) influenced the VCL and ALH of sperm coming from a 5-day storage ($p<.05$; Figure 3c,f). Moreover, ALH decreased in 1-day semen storage depending on CCK concentration, with a higher decrease of ALH when the highest concentration of CCK was used ($p<.05$). Any difference was detected for the rest of the parameters under these conditions (5 mmol/L HCO_3^-, $p>.05$; Figure 3a,b,d,e,j).

4 | DISCUSSION

Sperm capacitation is a complex event that induces physiological and biochemical changes in the sperm in order to acquire fertilizing capacity. Some of the modifications that occur during sperm capacitation are the reorganization of the plasma membrane, cholesterol loss, a rise in intracellular calcium, an increase in cAMP concentration, the phosphorylation of tyrosine residues, and the modification of sperm motility, among others (Brewis et al., 2005; Hunter & Rodriguez-Martinez, 2004; Stival et al., 2016; Yanagimachi, 1994; Zigo et al., 2020). In this study, sperm were incubated in a supporting capacitation medium containing 5 or 25 mmol/L HCO_3^-, with the addition of CCK protein. CCK and its receptors have been suggested to be involved in the regulation of protein tyrosine phosphorylation by modulating the uptake of HCO_3^- (Zhou et al., 2015), which is an essential factor for sperm capacitation. However, its effect on sperm motility has not been analysed yet.

Firstly, in this work, the expression of CCK1 and CCK2 receptors on boar testes has been demonstrated. This fact could

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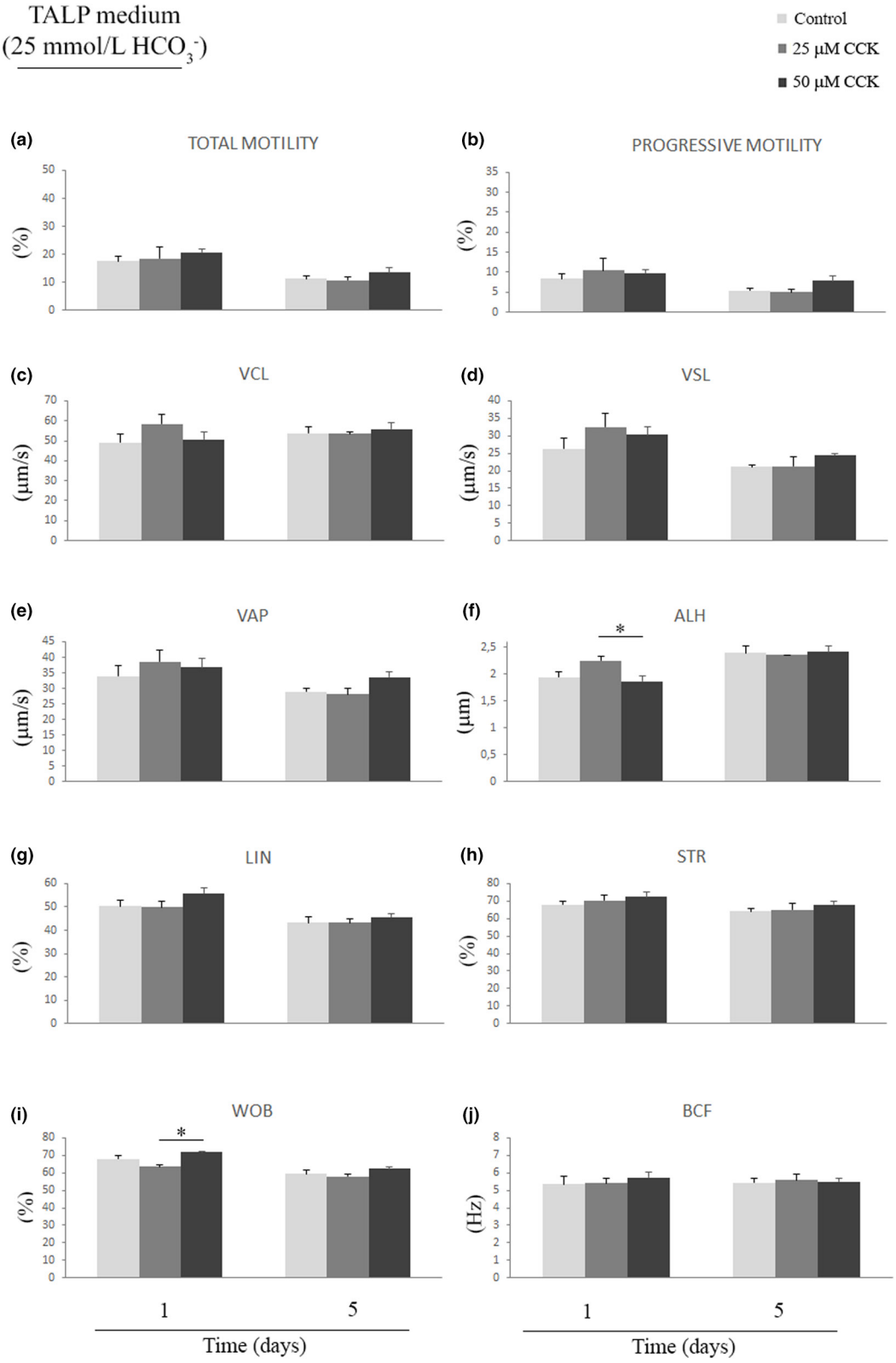


FIGURE 2 Bar chart showing the effect of CCK (Control-0 vs. CCK-25 vs. CCK-50) in sperm function (1-day or 5-day seminal doses) after incubation in capacitation conditions (TALP-25 mmol/L HCO_3^-). Asterisks between groups in the same period (1-day or 5-day seminal doses) indicate significant differences (*$p < .05$).

implicate that sperm also express these receptors, as previously reported for mouse sperm (Zhou et al., 2015). CCK activity is mediated by its receptors (Dufresne et al., 2006). After ligand activation, CCK1R and CCK2R stimulate calcium mobilization and the MAPK/ERK and cAMP/PKA signalling pathways (Wan et al., 2023; Zeng et al., 2020). Thus, the expression of CCK1R and CCK2R reinforces

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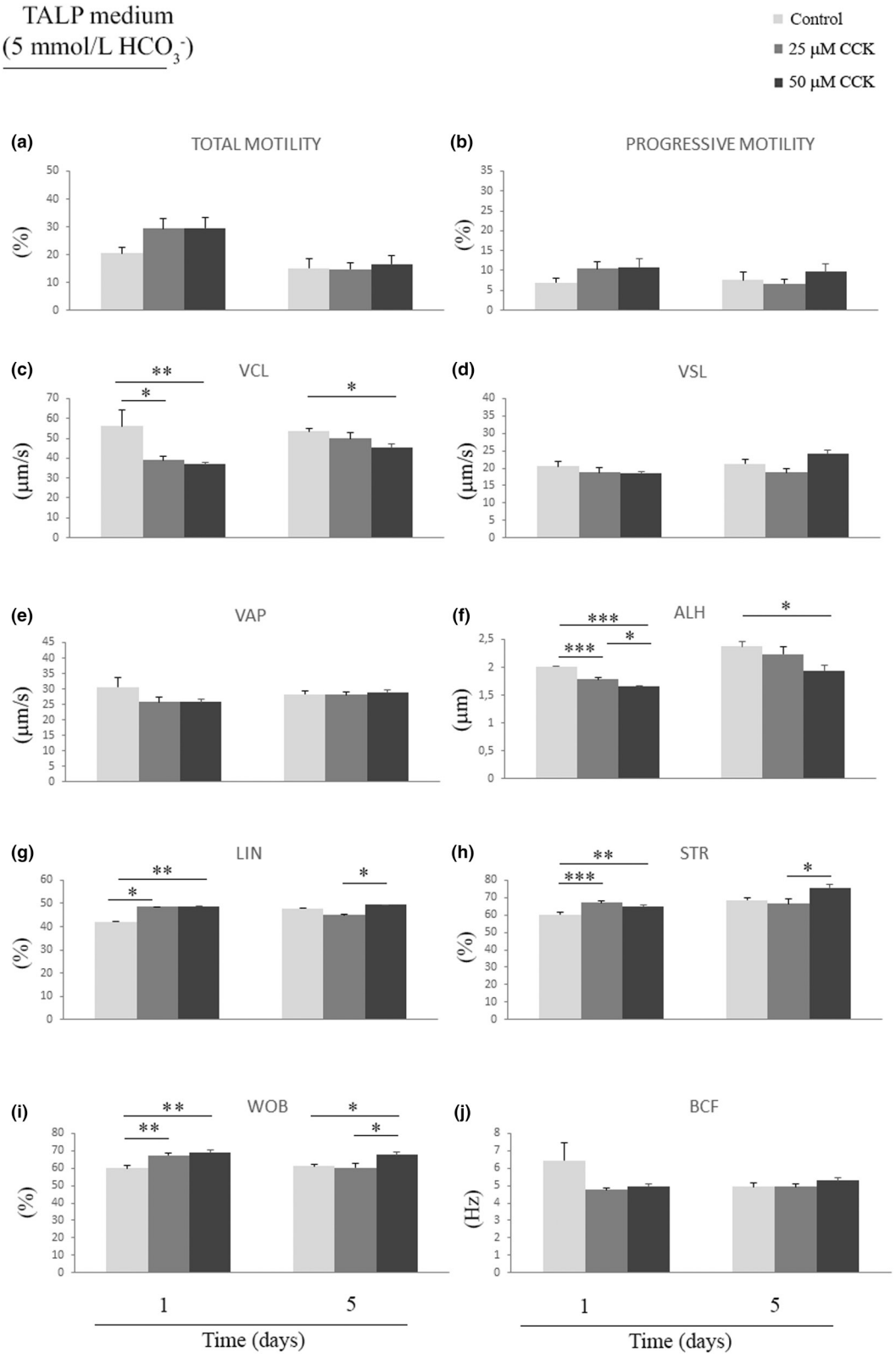


FIGURE 3 Bar chart showing the effect of CCK (Control-0 vs. CCK-25 vs. CCK-50) in sperm function (1-day or 5-day seminal doses) after incubation in capacitation conditions (TALP-5 mmol/L HCO_3^-). Asterisks between groups in the same period (1-day or 5-day seminal doses) indicate significant differences (*$p < .05$; **$p < .01$; ***$p < .001$).

the fact that oviductal CCK could regulate sperm capacitation after binding to them, modulating the uptake of HCO_3^-, also in porcine species.

Sperm capacitation media in porcine species usually contain 25 mmol/L HCO_3^-; however, a previous study demonstrated that a lower concentration is sufficient to trigger the in vitro sperm

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capacitation and increase the in vitro fertilization efficiency in porcine species (Soriano-Úbeda et al., 2019). In the present study, CCK concentration did not affect the proportion of motile sperm (percentage of motile sperm and progressive motility) in any condition but did affect kinetic parameters. The changes were mainly evident in 5 mmol/L HCO_3^-, and less pronounced in 25 mmol/L HCO_3^-, but any effect of CCK was observed when HCO_3^- was absent (0 mmol/L). These observations indicate that CCK has an effect only when HCO_3^- is present, confirming a previous study's results showing that tyrosine phosphorylation (a capacitation marker) induced by CCK was dependent on the presence of BSA and HCO_3^- in the medium (Zhou et al., 2015), which is in line with our results. Our data suggest that CCK is not important for motility activation, but it changes the type of movement. The presence of CCK in 5 mmol/L HCO_3^- induced an increase in LIN, STR, and WOB parameters, mainly in 1-day seminal doses. An increase in these parameters indicates sperm with more linear trajectories (Soriano-Úbeda et al., 2019), which has previously been related to the capacitation status in boars (García Herreros et al., 2005; Holt & Harrison, 2002). Moreover, this fact agrees with a decrease in VCL when CCK was present in the incubation media.

In light of our results, it was notable that the CCK effect was more pronounced in 1-day than in 5-day seminal doses. Furthermore, only the highest concentration of CCK (50 μm) had an effect on 5-day spermatozoa in comparison with the control (VCL, ALH). Accordingly, sperm storage provokes changes in spermatozoa membrane stability and capacitation dynamics, losing a specific response to capacitation conditions (Henning et al., 2012).

Interestingly, CCK did not affect sperm kinetics when incubated in capacitating media with 25 mmol/L HCO_3^-, in contrast to when less bicarbonate was used. It is known that sperm respond differently depending on bicarbonate concentration (Harrison et al., 1996; Soriano-Úbeda et al., 2019), and the maximum bicarbonate used may have saturated their kinetics and no effect was detected, as previously reported in other studies (Soriano-Úbeda et al., 2019).

In conclusion, the testes of porcine express CCK receptors, which confirms the implication of CCK protein during sperm capacitation under low bicarbonate concentration increasing the sperm linear trajectory, the effect observed being dependent on CCK concentration and spermatozoa ageing.

AUTHOR CONTRIBUTIONS

López-Úbeda, Luongo, Sòria-Monzó, and Grudzinska did the experiments. Abril-Sánchez and García-Vázquez did the statistics. Moros-Nicolás interpreted the results. Moros-Nicolás, Avilés, Izquierdo-Rico, and García-Vázquez wrote the manuscript. Izquierdo-Rico and García-Vázquez supervised the work. All the authors commented on the manuscript.

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CONFLICT OF INTEREST STATEMENT

None of the authors have any conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the results of this study are available upon reasonable request.

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