AMP-activated kinase, AMPK, is involved in the maintenance of the quality of extended boar semen during long-term storage.

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Abstract

Boar semen preservation for later use in artificial insemination is approached by semen dilution into an appropriate medium and by lowering the temperature to decrease spermatozoa metabolism. The AMP-activated kinase AMPK is a key cell energy sensor that controls cell metabolism and recently has been identified in boar spermatozoa. Our aim was to investigate the role of AMPK in spermatozoa functional parameters including motility, mitochondrial membrane potential, plasma membrane lipid organization, acrosome integrity and cell viability during long-term boar semen storage at 17ºC in Beltsville-Thawing Solution (BTS). Boar seminal doses were diluted in BTS in the presence or absence of different concentrations of AMPK inhibitor, compound C (1, 10 and 30µM) and evaluations were performed at 1, 2, 4, 7 or 10 days. Data demonstrate that AMPK becomes phosphorylated at Thr^{172} (active) during storage of boar semen reaching maximum levels at day 7. Moreover, AMPK inhibition during boar semen storage causes i) a potent inhibition of spermatozoa motility, ii) a reduction in the percentage of spermatozoa showing high mitochondria membrane potential, iii) a rise in the percentage of spermatozoa displaying high plasma membrane scrambling and iv) a loss of acrosomal membrane integrity. Our study suggests that AMPK activity plays an important role in the maintenance of the spermatozoa quality during long-term storage of boar semen.

Keywords: boar semen storage, AMPK, motility, viability, scrambling, acrosomal integrity.

1. Introduction

Pig production includes the development of seminal doses in liquid state, which is stored at 15-20°C for several days until semen is used for artificial insemination, AI [1]. To preserve spermatozoa for prolonged periods, the metabolic activity of these germ cells needs to be reduced and this is approached by semen dilution into an appropriate medium and by lowering the temperature [1]. Several commercial boar semen extenders have been proposed [2], although the most widely used extender is the Beltsville-Thawing Solution (BTS) developed by Pursel and Johnson [3] for thawing boar spermatozoa frozen in the pellet form, and later adapted for liquid storage by Johnson et al, [4]. One of the basic requirements in the development of extenders is to allow cooled spermatozoa an optimum use of energy sources present, mainly in the so called long-term extenders designed for semen storage for more than 3 days [2]. To date, the improvement of extenders for use in swine AI with cooled semen has been approached in studies based on i) the evaluation of the metabolic substrates used by spermatozoa to obtain energy or ii) the addition of compounds that minimize the alterations in the extender due to spermatozoa activity, such as oxidative stress, pH fluctuations or those that protect spermatozoa from cold shock [5]. However, as far as we know, no works have been performed aimed to study kinase-orchestrated intracellular pathways that regulate spermatozoa physiology during long-term boar semen storage at 17ºC.

Recently, we have identified for the first time the presence of the AMP-activated protein kinase (AMPK) in mammalian spermatozoa [6]. The AMPK is an enzyme conserved along the eukaryote kingdom that senses intracellular

AMP levels and controls cell metabolism [7] and therefore could act as a key molecule in the spermatozoa adaptation to the long-term storage process. In addition to allosteric activation by AMP, phosphorylation of Thr172 in its α subunit appears to be essential for AMPK activity because site directed mutagenesis of Thr 172 to alanine completely abolishes kinase activity [8,9]. Under energy limiting conditions or different cellular stresses AMPK becomes activated and phosphorylates several downstream substrates, leading to inhibition of ATP-consuming pathways (e.g. fatty acid and cholesterol synthesis) and simultaneously to activation of those ATP-generating pathways (e.g. fatty acid oxidation and glycolysis) [10,11].

As semen storage for long-term at 17°C is accompanied by intense metabolic and energetic changes in the spermatozoa, our objective was to study AMPK expression in these germ cells and its possible function in the maintenance of the quality of extended boar semen during long-term storage at 17 ºC in BTS.

2. Materials and Methods

2.1. Chemicals and Sources

Beltsville-Thawing Solution was from Minitub Iberica (Tarragona, Spain); Live/dead spermatozoa viability kit including both propidium iodine (PI) and SYBR-14 probes, M540 and YoPro-1 probes were purchased from Molecular Probes (Leiden, The Netherlands); compound C (6-[4-(2-Piperidin-1-ylethoxy) phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine) and FITC-PNA were from Sigma-Aldrich® (St Louis, MI, USA); anti-AMPKα antibody was from Cell Signaling (Beverly, CA); anti-P-Thr^{172}-AMPK antibody was from Santa Cruz Biotechnology (lot# H0210, Santa Cruz, CA, USA); coulter isoton II diluent from Beckman Coulter Inc. (Brea, CA, USA); JC-1 (5,5′,6,6′–tetrachloro-

105 1,1′,3,3′ tetraethylbenzymidazolyl carbocyanine iodine) probe, alexa Fluor 488
106 goat anti-rabbit IgG and ProLong gold antifade reagent with 4.6-diamidino- 2-
107 phenylindole hydrochloride (DAPI) from Life Technologies Ltd (Grand Island,
108 NY, USA); microscope slides coated with L-lysine from Electron Microscopy
109 Sciences (Hatfield, PA, USA).

110 **2.2. Boar semen collection and storage**

111 Fresh ejaculates samples from 6 boars (Duroc breed, 2-4 years of age)
112 housed at a commercial insemination station (Tecnogenext, S.L, Mérida,
113 Spain) were used as ejaculate donors. The boars were housed in individual
114 pens in an environmentally controlled (15-25ºC) building and received the
115 same diet. Artificial insemination using preserved liquid semen from these
116 boars demonstrated their fertility. Fresh ejaculates were collected with the
117 gloved hand technique and immediately placed in a water bath at 37ºC. After
118 collection, a computer-assisted sperm analysis system (CASA) (ISAS Psus®,
119 Proiser R+D S.L., Paterna, Valencia) was use for evaluation of sperm
120 characteristics (sperm concentration, motility and normal morphology). Only
121 ejaculates with at least 80% morphologically normal spermatozoa, 70% motile
122 spermatozoa and a total number of spermatozoa higher than 10×10^9 were
123 used. Immediately after collection, five sub-samples of each whole ejaculate
124 were diluted in BTS extender, to a final concentration of 35×10^6 cell/mL. The
125 extended semen from each sub-sample was then stored in plastic bottles in a
126 semen storage unit at 20-22ºC for approximately 60 min for transit to the
127 laboratory. Sperm sub-samples were treated as follows: a) by addition of
128 AMPK specific inhibitor, compound C (12.5mM stock solution in dimethyl
129 sulfoxide) at different final concentrations of 1, 10 or 30µM, b) by addition of

the highest DMSO concentration (0,24%) used to dilute compound C, and c) without any addition, untreated sub-sample (BTS). An aliquot of BTS sub-sample was analyzed for sperm quality (Day 0) before doses were stored in a refrigerated incubator (FOC 225 I, VELP Scientifica, Usmate, Italy) at 17ºC for 10 days. An aliquot of each sub-sample was removed and analyzed for sperm quality on Days 1, 2, 4, 7 and 10.

2.3. Western blotting

Spermatozoa under different treatments were centrifuged 20s at 7000*g*, washed with phosphate buffered saline (PBS) supplemented with 0.2mM Na_3VO_4 and then lysated in a lysis buffer consisting in 50mM Tris/HCl, pH 7.5, 150mM NaCl, 1% Triton X-100, 1% deoxycholate, 1mM EGTA, 0.4mM EDTA, protease inhibitors cocktail (Complete, EDTA-free) and 0.2mM Na_3VO_4, followed by sonication for 5s at 4ºC. After 20 minutes at 4ºC samples were centrifuged at 10000g (15 minutes, 4ºC) and the supernatant (lysate) collected for AMPK and phospho-AMPK analysis after evaluation of protein concentration by BioRad protein assay reagent. Proteins from porcine spermatozoa lysates were resolved by 10% SDS-PAGE and electro-transferred to nitrocellulose membranes. Western blotting was performed as previously described in these germ cells [6] using anti AMPKα (1:1000), anti phospho-Thr172-AMPKα (1:500) and anti GSK3β (1:2000) polyclonal antibodies as primary antibodies.

2.4. Immunolocalization of AMPKα in boar spermatozoa by immunofluorescence

Aliquots of 40µL of 4% paraformaldehyde-fixed sperm samples were spread onto poly-L-lysine coated microscope slides and were then left to air-dry.

Following three washings (5min in PBS), sperm samples were permeabilized by incubation for 10min at room temperature (RT) in PBS, pH 7.4 containing 0.25% (vol/vol) Triton X-100. Then, samples were washed three times with PBS and blocked through incubation with PBS including 0.1% (vol/vol) Tween-20 and 1% (wt/vol) BSA for 30min at RT. Incubation with primary antibody, AMPKα (1:100) diluted in blocking buffer was carried out overnight at 4ºC. Following the binding of specific antibody, samples were washed thoroughly with PBS and incubated with Alexa Fluor 488 goat anti-rabbit IgG (1:200). As negative controls, samples incubated with primary serum and without secondary antibody were run in parallel. Slides were gently washed with PBS and then incubated with 5µL of a commercial solution of 4.6-diamidino- 2-phenylindole hydrochloride (DAPI) 125 ng/mL as both a nuclear stain and an anti-fading mounting solution. Any excess of liquid was eliminated and coverslips were finally sealed with colorless nail polish and stored at 4ºC in the dark until microscope observation. Fluorescent images were obtained and evaluated using confocal laser scanning fluorescence microscopy (Fluoview FV1000; Olympus, Tokyo, Japan). The images obtained were processed using Adobe Photoshop CS5 (Adobe Systems, Mountain View, CA).

2.5. Evaluation of spermatozoa motility by Computer Assisted Sperm Analysis (ISAS®) system

Immediately after gentle mixing, 1mL of stored semen was taken from each bottle and examined for motility pattern using a CASA system (ISAS® program, Proiser R+D, Paterna, Valencia, Spain). Prior motility analysis, seminal doses were incubated with 5% CO_2 at 38.5ºC during 30min (Mini

Galaxy A, RS Biotech, United Kingdom). A total of 2µl of sample was placed in a prewarmed counting chamber (Leja®, Luzernestraat, The Netherlands). Sperm motility analysis is based on the examination of 25 consecutive digitalized images obtained from several fields using a 10X negative-phase contrast objective, and at least 300 spermatozoa per sample were analyzed. Images were taken with a time lapse of 1s. The number of objects incorrectly identified as spermatozoa was minimized.

After acquiring representative fields, the following sperm motility parameters were recorded: total motile spermatozoa (percentage of spermatozoa with an average path velocity > 10µm/s), progressively motile spermatozoa (percentage of spermatozoa with a straightness coefficient > 80%), VCL (curvilinear velocity in µm/s), VSL (straight-line velocity in µm/s), VAP (average path velocity in µm/s), LIN (linearity coefficient in %), STR (straightness coefficient in %), WOB (wobble coefficient in %) and ALH (amplitude of lateral head displacement in µm). For porcine spermatozoa the definition used for hyperactivated motility was spermatozoa with VCL ≥ 95µM/s, LIN ≤ 30%, ALH > 3,5µm and WOB ≤ 70% [12].

2.6. Flow cytometry analyses

Flow cytometry analyses were performed using a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter Ltd.) The fluorophores were excited by a 200mV argon ion laser operating at 488nm. A total of 10000 gated events based on the forward scatter and side scatter of the sperm population recorded in the linear mode) were collected per sample with a running rate of approximately 500 events/s. Fluorescence data were collected in the logarithmic mode and

analyzed using a FACStation^{TM} and EXPO^{TM} 32 ADC software (Beckman Coulter, Inc.)

2.7. Analysis of spermatozoa viability by flow cytometry

As described previously [13] fluorescent staining using the LIVE/DEAD Sperm Viability Kit was performed to assess porcine spermatozoa viability. Briefly, 5µl of SYBR-14 (2µM) and 10µl of propidium iode (PI 5µM) were added to 500µL of diluted semen sample in isotonic buffered diluent coulter isoton II and incubated 20 min at room temperature in the darkness. After incubation, cells were analyzed by flow cytometry and the percentage of viable spermatozoa is expressed as the average of the percentage of SYBR14-positive and propidium iodide-negative spermatozoa.

2.8. Evaluation of the status of organization of spermatozoa plasma membrane.

Spermatozoa plasma membrane lipid architecture was assessed by staining with merocyanine 540 (M540) and plasma membrane permeability by staining with YoPro-1. Aliquots of 100µl of each semen sample (35 x 10^6 cells/mL) were diluted in 400µl of isotonic buffered diluent containing 75nmol/L YoPro-1, which were then mixed and incubated at 38ºC for 15min. Just before analysis, M540 was added to each sample to a final concentration of 2 µmol/L, incubated for 2min and remixed before flow citometry analysis. Labeled spermatozoa were categorized as (1) viable cells with low plasma membrane scrambling (YoPro-1^-/M540); (2) viable cells with high plasma membrane scrambling (YoPro-1^-/$M540^+$); or (3) non-viable cells with altered permeability (Yo-Pro-1^+). The percentage of viable cells with high plasma membrane scrambling is expressed as the average percentage.

2.9. Evaluation of the integrity of acrosome in boar spermatozoa by flow cytometry.

The acrosomal status of spermatozoa was assessed after staining the spermatozoa with phycoerythrin (PNA-FITC), as a marker for acrosome status, and PI. Aliquots of 100µl of each semen sample (35 x 10^6 cells/mL) were incubated at room temperature in the dark for 5min with 1µg/mL PNA-FITC and 6µmol/L PI. Just before analysis, 400µl of isotonic buffered diluent was added to each sample and remixed before flow cytometry analysis. Cells were analyzed and the percentage of spermatozoa with acrosome damaged or reacted is expressed as the average percentage of PNA-positive and PI-negative spermatozoa.

2.10. Analysis of mitochondrial membrane potential by flow cytometry.

Mitochondrial membrane potential variations were evaluated using the specific probe JC-1. This lipophilic cationic fluorochrome JC-1 is present as protomeric aggregates in mitochondria with high membrane potential that emit in orange (590nm), whereas in mitochondria with low membrane potential, JC-1 is present as monomers that emit in green (525nm) when excited at 488nm. From each sperm sample, 100µl (35 x 10^6 cells/ml) were diluted in 400µl of isotonic buffered diluent containing 0.15mmol/L JC-1 and then mixed and incubated at 38ºC for 30min. The samples were remixed before flow cytometry analysis. The percentage of orange stained cells, which represents the population of germ cells with high mitochondrial membrane potential (hMMP), was recorded and expressed as the average of several experiments.

2.11 Statistical Analysis

The mean and standard error of the mean were calculated for descriptive statistics, whenever it was possible. The effect of treatment and incubation time on spermatozoa parameters was assessed using a General Linear Model. All analyses were performed using SPSS v15.0 for Windows software (SPSS Inc. Chicago, IL). The level of significance was set at $p < 0.05$.

3. Results

3.1. Effect of long-term semen storage in BTS at 17ºC on boar spermatozoa viability, mitochondrial membrane potential, plasma membrane organization and acrosome integrity.

As we have shown previously [14], plasma membrane lipid organization is an extremely sensitive parameter to long-term boar semen storage at 17ºC. This preservation method leads to a statistically significant time-dependent increase in the percentage of boar spermatozoa with high MD540 fluorescence (9.8±1.3% at day 0 and rising up to 25.4±4.8% at day 10), which is indicative of a higher degree of plasma membrane scrambling with increasing semen storage time (Table 1). In parallel, storage of boar semen causes a slight time-dependent increase in the percentage of spermatozoa showing damaged or reacted acrosome, reaching maximum levels of compromised acrosome integrity at day 10 (Table 1), although this increase was not statistically different compared with day 0. Regarding the effect of long-term storage in boar spermatozoa viability, the percentage of viable germ cells with an intact plasma membrane is maintained until day 7 (Table 1), which confirms our previous results in this specie [14]. However, a small but significant decrease in the percentage of viable spermatozoa occurs at day 10 of storage (Table 1). The first 2 days of semen storage cause a significant increase in the percentage of spermatozoa showing high mitochondrial membrane potential, then decline gradually over the time reaching at day 10 similar values to day 0 (Table 1). The possible effect of solvent DMSO (0,24%) in the above-mentioned spermatozoa parameters was analyzed in parallel samples. BTS including 0.24% DMSO does not affect spermatozoa

viability, mitochondrial membrane potential, plasma membrane scrambling or the integrity of acrosome during semen storage at 17ºC for 10 days compared with semen stored in BTS alone (Table 1).

3.2 Effect of long-term semen storage in BTS at 17ºC on boar spermatozoa motility.

According to the effect described previously [14], storage of semen in BTS at 17ºC causes a statistically significant decrease in the percentage of motile spermatozoa starting from day 7, without any significant effect in other motility parameters analyzed (Table 2). The solvent DMSO (0,24%) does not significantly modify any spermatozoa motility parameters evaluated during semen storage (Table 2).

3.3. Identification and localization of AMP-activated kinase, AMPK, in fresh boar ejaculates.

The expression of AMPK in fresh boar ejaculates was investigated by Western blotting analysis using an antibody against the catalytic α subunit of AMPK as primary antibody. Two cross-reactive bands are detected in spermatozoa lysates from fresh ejaculates of six different boars at the correct molecular weight of the α subunit of AMPK (Figure 1A). Negative control for this antibody was performed omitting the primary antibody and probing the blot with secondary antibody (anti-rabbit-HRP) only. Results show that no band is detected with the secondary antibody and confirm that bands visualized are due to the AMPKα antibody used (data not shown).

The expression of AMPK protein was investigated by indirect immunofluorescence in boar spermatozoa using the same antibody against the catalytic α subunit of AMPK. Results demonstrate that AMPK protein is

309 highly expressed in boar spermatozoa stored at 17ºC in BTS and is mainly
310 localized at the entire acrosome of the spermatozoa head and in the midpiece
311 of the flagellum, as seen in Figure 1B.

312 **3.4. Spermatozoa AMPK becomes highly phosphorylated at Thr^{172}**
313 **(active) during boar semen storage at 17ºC in BTS.**

314 During long-term boar semen storage the level of phosphorylation of AMPK in
315 Thr^{172} was analyzed as assessment of its enzymatic activity using the specific
316 antibody anti-phospho-Thr^{172}-AMPKα, as we have previously described in
317 these germ cells incubated in different media [6]. As shown in Figure 2, one
318 cross-reactive band is detected at the correct molecular weight with anti-
319 phospho-Thr^{172}-AMPKα antibody. Our results show that in fresh boar
320 ejaculates (day 0) phosphorylation of AMPK is not detected, which indicates
321 that under these conditions the majority of AMPK is not active. However, just
322 one day of storage clearly leads to the threonine phosphorylation (activation)
323 of AMPK which is further and significantly enhanced during long-term storage
324 at 17ºC in BTS in a time dependent manner, reaching maximum levels at day
325 7 of storage (Figure 2). A loading control of protein is showed in lower panel of
326 Figure 2 using an anti-GSK3β antibody, as we have previously shown that
327 amount of this protein in boar spermatozoa does not change under our
328 experimental conditions [15].

329 **3.5. Effects of the AMPK inhibitor, Compound C, on the viability and the**
330 **mitochondrial membrane potential of spermatozoa during long-term**
331 **boar semen storage at 17ºC in BTS**

332 A widely used inhibitor of the AMPK activity in somatic cells is the compound
333 C (CC), a cell-permeable pyrrazolopyrimidine molecule that acts as a potent,

selective, reversible, and ATP-competitive inhibitor of AMPK (K_i = 109 nM in the presence of 5 µM ATP and the absence of AMP). In boar spermatozoa, we have confirmed that CC (30 µM) effectively blocks the phosphorylation (activation) of AMPK that occurs under spermatozoa physiological conditions without any side effect in these germ cells [6].

The storage at 17ºC of boar semen during long-term in the presence of different concentrations of CC (1, 10 and 30µM) does not affect the percentage of viable spermatozoa with an intact plasma membrane, when compared with BTS alone (Table 3). However, the presence of the highest concentration used 30 µM CC causes a significant decrease in the percentage of spermatozoa displaying high mitochondrial membrane potential starting at 7 days of semen storage (Table 3).

3.6. Effects of the AMPK inhibitor on the lipid organization of spermatozoa plasma membrane and in the acrosomal integrity during boar semen storage at 17ºC in BTS.

Next, we aimed to evaluate whether AMPK plays a role in the regulation of the lipid organization of spermatozoa plasma membrane during semen storage at 17ºC in BTS, as it is a well-known parameter that indicates the functional status of spermatozoa. Our results shows that AMPK inhibitor (CC 30µM) causes a significant increase in the percentage of spermatozoa showing higher disorganization of plasma membrane after 4 days of semen storage (Figure 3A), reaching maximum values of 62.6±7.1% at day 10 (Figure 3A). This CC-induced rise in the percentage of spermatozoa showing higher plasma membrane scrambling is accompanied by a statistically significant increase in the percentage of spermatozoa presenting altered acrosomal

membrane at days 7 and 10 of semen storage in the presence of CC (30µM) (Figure 3B).

3.7. Effect of AMPK inhibition in spermatozoa motility during boar semen storage at 17ºC in BTS

To evaluate the effect of the AMPK inhibition in spermatozoa motility parameters during semen storage, boar seminal doses were diluted in BTS in the presence or absence of different concentrations of CC (1, 10 and 30µM). After 1, 2, 4, 7 or 10 days of storage at 17ºC, seminal doses were incubated with 5% CO_2 at 38.5ºC during 30min to ensure motility and then motility parameters were evaluated.

In the short-term of semen storage (1-2 days), inhibition of AMPK with CC causes a slight but statistically significant increase in the percentage of motile spermatozoa (Figure 5), without affecting the curvilinear velocity VCL (Figure 6). Moreover, during these 2 days of storage the inhibition of AMPK leads to a significant increase in both the linearity (LIN) of spermatozoa movement, as well as in the percentage of spermatozoa showing progressive movement. At the same time, CC treatment (30µM) causes a clear and statistically significant decrease in the percentage of spermatozoa showing hyperactivated motility (Table 4).

In the long-term, after 4 days of semen storage, inhibition of AMPK with different concentrations of CC (1, 10 and 30µM) causes a clear and significant decrease in any spermatozoa velocity analyzed, VCL (Figure 6), VAP and VSL (data not shown). In addition, treatment with 30µM CC caused a reduction in the percentage of motile spermatozoa at day 10 of semen storage (Figure 5), accompanied by a recovery to initial data (day 0) of the values of

LIN coefficient, percentage of spermatozoa with progressive movement and percentage of spermatozoa having hyperactivated motility (Table 4).

4. Discussion

Long-term storage of boar ejaculates produces a gradual lost of the ability of spermatozoa to fertilize when preserved semen doses are later used in artificial insemination, especially if the number of fertile spermatozoa falls below a certain threshold level [16]. During storage at 15-17ºC, boar spermatozoa undergo several changes, including among others: decreased motility [17], increase in the proportions of prematurely capacitated-like and acrosome damaged spermatozoa [18-20] and decrease in ATP concentration [21]. Previous studies have demonstrated that preservation of boar spermatozoa for prolonged periods requires a decrease in their metabolic activity, which is carried out by lowering temperature to 15-17ºC and diluting into an appropriate medium [1,2]. The control of cell metabolism in spermatozoa is achieved by dynamic mechanisms able to adapt to environmental changes and related with cellular structures such as mitochondria or plasmalemma [22]. Therefore, regulation of the energy levels is of essential importance in the understanding of spermatozoa function during long-term storage at 17ºC. In mammalian tissues the protein AMPK controls metabolism [23,24] by activating metabolic pathways that produce ATP and simultaneously by inhibiting those pathways that consume ATP [25, 26].

In a recent work, we have shown for the first time that the metabolic sensor AMPK is expressed in spermatozoa and that AMPK phosphorylation (activation) is dependent of the temperature [6]. Moreover, the present study shows that the metabolic sensor kinase AMPK is localized at the entire

409 acrosome at relatively high level and in the midpiece of flagellum in boar
410 spermatozoa stored at 17ºC in BTS. Furthermore, our results demonstrate
411 that AMPK becomes phosphorylated at Thr^{172}, and therefore subsequently
412 activated, during boar semen storage at 17ºC in BTS. The phosphorylation
413 level of AMPK varies with the time of storage with more than 10 fold increase
414 after 4 days and a maximum level at 7 days. This increase in the Thr^{172}-AMPK
415 phosphorylation might be the consequence of a fall in ATP concentration
416 during boar semen storage at 17ºC, as demonstrated by Gogol et al [21]. In
417 fact, the fall in ATP content is accentuated after 4 days of storage [21], which
418 correlates with the marked increase in AMPK activity observed in our work.
419 Thus it seems logical to expect that a fall in the spermatozoa concentration of
420 ATP lead to spermatozoa AMPK activation and the consequent activation of
421 the metabolic pathways that produce ATP and simultaneous inhibition of
422 those pathways that consume ATP. Our results point to a possible role of
423 AMPK activity in the maintenance of the spermatozoa mitochondrial
424 membrane potential and the subsequent ATP production during boar
425 spermatozoa long-term storage at 17ºC in BTS by several reasons: 1) AMPK
426 inhibition by CC treatment leads to a statistically significant decrease in the
427 percentage of spermatozoa presenting high mitochondrial membrane
428 potential, 2) the intracellular localization of AMPK, which is found in the
429 midpiece of the spermatozoa flagellum, where mitochondria are localized and
430 helically arranged; 3) the higher increase in active phospho-Thr^{172}-AMPK
431 correlated with the decrease in the ATP concentration observed in boar
432 spermatozoa storage at 17ºC [21] and, finally, 4) in the case that a fall in the
433 mitochondrial membrane potential might occur during the process of boar

434 spermatozoa death, as it has been previously shown in human spermatozoa
435 [27], then, it could be an explanation for the fall in viability of spermatozoa
436 when AMPK is inhibited by treatment with CC during long-term storage.
437 As it has been described previously [14,17], our results show that storage of
438 extended boar semen for long time causes a clear reduction of the percentage
439 of motile spermatozoa. The fact that CC treatment causes a clear and
440 significant reduction of the percentage of motile spermatozoa and also in their
441 velocity after 4 days of semen storage at 17ºC implies that AMPK inhibition
442 induces a potent increase in the number of motionless spermatozoa together
443 with a clear reduction in the velocity average VAP of the remaining motile
444 spermatozoa. Recently, we have shown that AMPK activity is necessary for
445 optimal boar spermatozoa motility under physiological conditions [6], which is
446 in agreement with our results obtained in extended boar semen during long-
447 term storage at 17ºC. Having in mind the energy-regulating role of AMPK in
448 somatic cells, it is logical to assume that AMPK activity may play a role in
449 those spermatozoa functions that are particularly dependent of the energy
450 levels, such as motility. Thus, as AMPK plays a central role in the
451 maintenance of cell energy levels by regulating among others pathways the
452 glycolysis [28], it is reasonable to assume that the decrease in the percentage
453 of spermatozoa presenting high mitochondrial membrane potential after
454 AMPK inhibition could additionally account for the inhibition of spermatozoa
455 motility in extended boar semen during long-term storage at 17ºC. However,
456 we cannot exclude a possible non-metabolic effect of the AMPK pathway that
457 could be involved in the control of spermatozoa motility in a parallel or
458 synergistic way, as AMPK is a serine/threonine kinase with several known

459 downstream substrates and therefore may regulate processes outside of cell
460 metabolism [29].
461 It has been clearly described that sperm handling for assisted reproduction,
462 such as dilution, cooling or long-term storage, affect the sperm surface mainly
463 in the acrosomal region [18]. Therefore, during boar semen storage the
464 spermatozoa plasmalemma needs to be stabilized to avoid an early
465 capacitation or a loss in the integrity of acrosomal membrane [18]. Our results
466 suggest that spermatozoa AMPK activity contributes to the stability of the
467 plasmatic and acrosomal membranes as: 1) The spermatozoa plasma
468 membrane region surrounding the acrosome is especially modified during
469 capacitation and also during sperm handling for artificial insemination [18].
470 The intracellular localization of AMPK in the head of spermatozoa is confined
471 to this region, 2) activation of AMPK is sensitive to the liquid storage of sperm,
472 our results show a clear increase in the phosphorylation of AMPK (active)
473 correlative to storage time, 3) inhibition of AMPK potentiates the spermatozoa
474 plasma membranes alterations observed during boar semen storage such as
475 the increase in plasmalemma scrambling and the deterioration of the integrity
476 of acrosomal membrane.
477 Johnson et al [1] described that the structural and functional changes of
478 spermatozoa connected with liquid storage in boar semen resemble a natural
479 aging process that precedes the aging of spermatozoa after artificial
480 insemination waiting to be released from the lower part of the isthmus in
481 response to the ovulation [1]. Our results point out that this postulated aging
482 process would activate AMPK in boar spermatozoa and that AMPK is an

important kinase for improving the quality of extended boar semen mainly after long-term storage.

6. Conclusions

In summary, the present study demonstrates that AMPK becomes phosphorylated at Thr^{172} (active) during refrigerated liquid storage of boar semen. Our results shows that AMPK inhibition during boar semen storage at 17ºC causes a potent inhibition of spermatozoa motility, a reduction in the percentage of spermatozoa showing high mitochondria membrane potential and an increase in the both percentage of spermatozoa showing high plasmalemma scrambling and in the loss of acrosomal membrane integrity. Our study points to an important role of AMPK in maintenance of the quality of extended boar semen during long-term storage at 17ºC, and therefore it is important to understand what intracellular or extracellular factor(s) trigger the activation of AMPK and how the regulation of this kinase can be used to improve the quality and time of storage of boar semen doses for assisted reproduction.

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8. Figure Legends

Figure 1. Identification and immunolocalization of AMPK in boar spermatozoa.

A: spermatozoa isolated from fresh ejaculates of different boars (numbered) were lysed, subjected to SDS-PAGE, electrotransferred to nitrocellulose and western blotting performed using anti-AMPKα as primary antibody as described in Materials and Methods. The antibody against AMPKα recognized two bands close to 64kD. Molecular weight markers are indicated at the right side. A representative film including ejaculates from 6 boars is shown (n=12). B: fresh spermatozoa from different boars were pooled and fixed in 4% paraforlmaldehide and immunostaining was performed using antibody against the catalytic subunit of AMPKα. The immunofluorescence was visualized in a confocal microscope and a representative image is shown at the left panel, whereas Normaski optic is shown at the right panel. Arrows show AMPKα immunolocalization. Scale bar is 10µm.

Figure 2. Phosphorylation of AMPK at Thr^{172} in spermatozoa from extended boar semen during long-term storage at 17ºC.

Boar semen was stored in BTS at 17ºC for 10 days. At the indicated time spermatozoa were lysed and proteins (20µg) from lysates were analyzed by western blotting using anti-phospho-Thr^{172}-AMPKα as primary antibody, as described in Materials and Methods. Arrow indicates the crossreactive band of phospho-Thr^{172}AMPK, recognized by the anti-AMPKα (upper film). Loading controls were performed for each experiment in the same membranes (with different time of chemiluminescence exposure) using anti-GSK3β antibody (lower film). This image is representative of another six performed in duplicate. The *bottom panel* shows the quantification of the intensity of

phospho-Thr^{172}AMPK obtained in each sample. Values are the mean ± standard error of the mean (n=12) expressed as relative arbitrary units after scanning the films. An asterisk indicates significant differences when compared with day 0 ($p<0.05$).

Figure 3. Effect of spermatozoa AMPK inhibition with different concentrations of compound C in the plasma membrane scrambling and acrosome reaction in extended boar semen during long-term storage at 17ºC

Boar semen was stored at 17ºC during 10 days in BTS in presence or absence of the AMPK inhibitor CC (1, 10 and 30µM) or DMSO (0,24%). *Figure A*, the level of lipid disorganization of spermatozoa plasma membrane was measured by flow cytometry as described in Material and Methods using merocyanine M540 as a probe. Spermatozoa population exhibiting plasma membrane scrambling M540^{+} is expressed as percentage of the total live spermatozoa (YoPro-1^{-}). Results are expressed as the mean ± standard error of the mean (n=12). Values with asterisk are statistically different from control (BTS) value ($P<0.05$). *Figure B*, Acrosomal integrity was measured by flow cytometry as described in Material and Methods using PNA-FITC as a probe. Spermatozoa population PNA^{+}/PI^{-} is expressed as percentage of total spermatozoa. Results are expressed as the mean ± standard error of the mean (n=12). Values with asterisk are statistically different from control values considered as samples stored in BTS alone ($P<0.05$).

Figure 4. Effect of AMPK inhibition with different concentrations of compound C in the percentage of motile spermatozoa from extended boar semen during long-term storage at 17ºC

Boar semen was stored at 17ºC during 10 days in BTS in presence or absence of the AMPK inhibitor CC (1, 10 and 30µM) or DMSO (0,24%). The

percentage of motile spermatozoa was measured by CASA (ISAS®) system as described in Materials and Methods. Results are mean ± standard error of the mean (n=12). Values with asterisk are statistically different from control values considered as samples stored in BTS alone (P<0,05).

Figure 5. Effect of AMPK inhibition with different concentrations of compound C in the curvilinear velocity of spermatozoa from extended boar semen during long-term storage at 17ºC

Boar semen was stored at 17ºC during 10 days in BTS in presence or absence of the AMPK inhibitor CC (1, 10 and 30µM) or DMSO (0,24%). Curvilinear spermatozoa velocity (VCL µM/s) was measured by CASA (ISAS®) system as described in Materials and Methods. Results are mean ± standard error of the mean (n=12). Values with asterisk are statistically different from control values considered as samples stored in BTS alone (P<0,05).

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Table 1. Effect of long-term semen storage in BTS at 17ºC on boar spermatozoa viability, mitochondrial membrane potential, membrane fluidity and acrosome integrity.

Storage	Cell Viability (%)		High Mitochondrial Membrane Potential (%)		Plasma Membrane Fluidity (%)		Acrosome Damaged (%)	
Day 0	92,94±0,33		66,57±3,35		9,8±1,3		6,4±1,0	
Treatment	BTS	DMSO	BTS	DMSO	BTS	DMSO	BTS	DMSO
Day 1	90,86 ± 0,88	91,75 ± 0,51	80,93* ± 1,97	75,93* ± 2,45	10,0 ± 1,2	10,5 ±1,3	6,5 ± 1,1	6,9 ± 1,0
Day 2	91,05 ± 0,78	91,18 ± 0,50	78,72* ± 1,77	76,17* ± 1,76	10,4 ± 1,2	10,5 ± 1,0	6,3 ± 0,9	6,6 ± 1,0
Day 4	89,53 ± 1,16	91,13 ± 0,67	75,77 ± 2,98	72,58 ± 4,33	12,0 ± 1,0	11,7 ± 1,4	6,9 0,8±	7,1 ± 1,0
Day 7	88,81 ± 1,24	90,09 ± 4,00	70,11 ± 4,96	65,36 ± 2,99	16,5 ±2,0	15,6 ±1,8	7,9 ± 1,0	7,5 ± 0,9
Day 10	85,03* ± 1,80	82,95* ± 0,37	67,68 ± 3,84	65,37 ± 5,00	25,4* ± 4,8	24,6* ± 2,1	9,3 ± 1,4	9,6 ± 1,4

Boar seminal doses were preserved at 17ºC during 10 days in BTS in presence or absence of DMSO (0,24%). Percentages of cell viability, high mitochondrial membrane potential, plasma membrane fluidity and acrosomal integrity of spermatozoa were measured by flow cytometry as described in Materials and Methods. Results are Mean ± standard error of the mean (SEM) (n=12). Within a column, values with asterisk are statistically different from day 0 ($P<0.05$). There are not statistical differences between treatments (BTS and BTS+DMSO).

Table 2. Effect of long-term semen storage in BTS at 17ºC on boar spermatozoa motility parameters.

Storage	Motile (%)		Progressively Motile (%)		VCL (µm/s)		VAP (µm/s)		Linearity Coefficient (%)		Hyperactivated Motility (%)	
Day 0	81,7 ± 2,1		41,7 ± 6,0		77,8 ± 4,1		60,8 ± 2,2		54,6 ± 4,0		6,8 ± 2,6	
Treatment	BTS	DMSO	BTS	DMSO	BTS	DMSO	BTS	DMSO	BTS	DMSO	BTS	DMSO
Day 1	73,6 ± 3,0	66,4 ± 4,4	37,1 ± 5,2	38,5 ± 5,7	79,3 ± 4,1	75,8 ± 2,5	59,7 ± 3,0	55,8 ± 2,8	49,9 ± 3,2	50,8 ± 3,17	7,8 ± 2,6	8,7 ± 2,2
Day 2	69,7 ± 4,1	68,8 ± 3,6	41,4 ±4,8	42,3 ± 4,9	76,6 ± 3,6	76,6 ± 2,5	57,5 ± 2,7	57,0 ± 3,0	52,0 ± 3,2	52,6 ± 3,5	8,1 ± 2,1	8,3 ± 2,8
Day 4	73,6 ± 5,0	69,9 ± 4,0	49,7 ± 5,2	50,5 ± 4,9	79,0 ± 3,3	76,5 ± 2,9	61,5 ± 2,5	58,7 ± 3,0	57,6 ± 3,2	57,9 ± 3,04	6,1 ± 2,7	6,8 ± 1,9
Day 7	63,6* ± 6,1	68,2* ± 2,7	51,6 ± 3,3	54,4 ± 4,6	70,9 ± 4,1	73,7 ± 2,9	53,2 ± 3,7	56,3 ± 2,9	57,0 ± 1,9	58,6 ± 2,7	4,6 ± 1,6	5,4 ± 1,2
Day 10	55,9* ± 7,1	59,4* ± 4,6	45,8 ± 2,9	54,5 ± 4,8	78,0 ± 3,8	77,1 ± 3,3	55,7 ± 3,8	54,8 ± 3,8	52,2 ± 2,3	55,1 ± 3,1	6,8 ± 0,7	7,1 ± 1,2

Boar seminal doses were preserved at 17ºC during 10 days in BTS in presence or absence of DMSO (0,24%). Spermatozoa motility parameters were measured by CASA (ISAS®) system as described in Materials and Methods. Results are Mean ± standard error of the mean (SEM) (n=12). Within a column, values with asterisk are statistically different from day 0 ($P<0.05$). There are not statistical differences between treatments (BTS and BTS+DMSO).

Table 3. Effect of the AMPK inhibitor compound C (CC) on boar spermatozoa viability and mitochondrial membrane potential during long-term semen storage in BTS at 17ºC.

Storage	Cell Viability (%)				High Mitochondrial Membrane Potential (%)			
Day 0	92,94±0,33				66,57±3,35			
Treatment	BTS	CC-1	CC-10	CC-30	BTS	CC-1	CC-10	CC-30
Day 1	90,86 ± 0,88	92,28 ± 0,50	91,23 ± 0,50	86,98 ± 0,82	80,93 ± 1,97	78,11 ± 1,89	79,33 ± 1,68	77,05 ± 2,20
Day 2	91,05 ± 0,78	92,30 ± 0,59	91,41 ± 0,71	87,41 ± 1,03	78,72 ± 1,77	76,43 ± 1,95	77,46 ± 4,64	74,84 ± 1,69
Day 4	89,53 ± 1,16	91,87 ± 0,42	90,33 ± 0,78	86,14 ± 0,83	75,77 ± 2,98	72,41 ± 2,47	76,19 ± 1,90	77,05 ± 1,41
Day 7	88,81 ± 1,24	89,83 ± 0,63	88,40 ± 0,78	84,91 ± 0,61	70,11 ± 4,96	68,89 ± 2,21	67,86 ± 2,15	59,32* ± 5,17
Day 10	85,03 ± 1,80	79,89 ± 4,58	84,11 ± 2,25	80,87 ± 1,61	67,68 ± 3,84	64,93 ± 5,57	67,05 ± 3,54	57,76* ± 5,60

Boar seminal doses were preserved at 17ºC during 10 days in BTS in presence or absence of different concentrations of compound C, CC (1, 10 and 30µM). Percentages regarding germ cells viability and spermatozoa with high ΔΨ mitochondrial were measured by flow cytometry as described in Materials and Methods. Results are expressed as Mean ± standard error of the mean (SEM) (n=12). Within a row, values with asterisk are statistically different from BTS alone (P<0,05).

Table 4. Effect of the AMPK inhibitor compound C (CC) on spermatozoa progressive motility during long-term boar semen storage in BTS at 17ºC.

Storage	Linearity Coefficient LIN (%)				Progressive Motility Spermatozoa (%)				Hyperactivated Motility (%)			
Day 0	54,6 ± 4,0				41,7 ± 6,0				6,8 ± 2,6			
Treatment	BTS	CC-1	CC-10	CC-30	BTS	CC-1	CC-10	CC-30	BTS	CC-1	CC-10	CC-30
Day 1	49,9 [a] ± 3,2	50,4 [a] ±2,7	58,1 [a] ±2,7	65,8 [b] ±2,5	37,1 [a] ± 5,2	36,9 [a] ±4,1	47,9 [a] ±4,6	61,9 [b] ±4,1	7,8 [a] ± 2,6	7,6 [a] ±1,9	4,3 [a] ±1,1	2,7 [b] ±0,7
Day 2	52,0 [a] ± 3,2	50,9 [a] ±3,1	59,1 [a] ±2,8	65,8 [b] ±2,5	41,4 [a] ±4,8	38,7 [a] ±4,7	49,5 [a] ±4,7	62,5 [b] ±4,2	8,1 [a] ± 2,1	9,3 [a] ±2,6	4,2 [a] ±1,0	2,7 [b] ±0,7
Day 4	57,6 ± 3,2	58,0 ±2,4	62,3 ±3,3	66,3 ±2	49,7 [a] ± 5,2	50,7 [a] ±4,0	59,0 [a] ±7,1	67,7 [b] ±3,0	6,1 ± 2,7	5,7 ±1,2	4,4 ±1,3	2,4 ±0,6
Day 7	57,0 ± 1,9	56,7 ±2,4	59,3 ±2,4	56,3 ±3,1	51,6 ± 3,3	49,7 ±3,5	56,0 ±3,9	59,4 ±4,3	4,6 ± 1,6	5,3 ±0,6	3,6 ±0,7	4,1 ±1,0
Day 10	52,2 ± 2,3	56,1 ±1,8	55,4 ±1,9	46,7 ±3,4	45,8 ± 2,9	54,4 ±2,9	56,0 ±3,0	44,7 ±7,6	6,8 ± 0,7	6,1 ±1,1	4,9 ±0,6	7,3 ±1,42

Boar seminal doses were preserved at 17ºC during 10 days in BTS in presence or absence of compound C (1, 10 and 30µM). Linearity coefficient (LIN) and percentage of spermatozoa presenting progressive motility and hyperactivated motility spermatozoa were measured by CASA (ISAS®) system as described in Materials and Methods. Results are Mean ± standard error of the mean (SEM) (n=12). Within a row, values with different superscripts are statistically different from control value (BTS, $P<0,05$).

Figure 1

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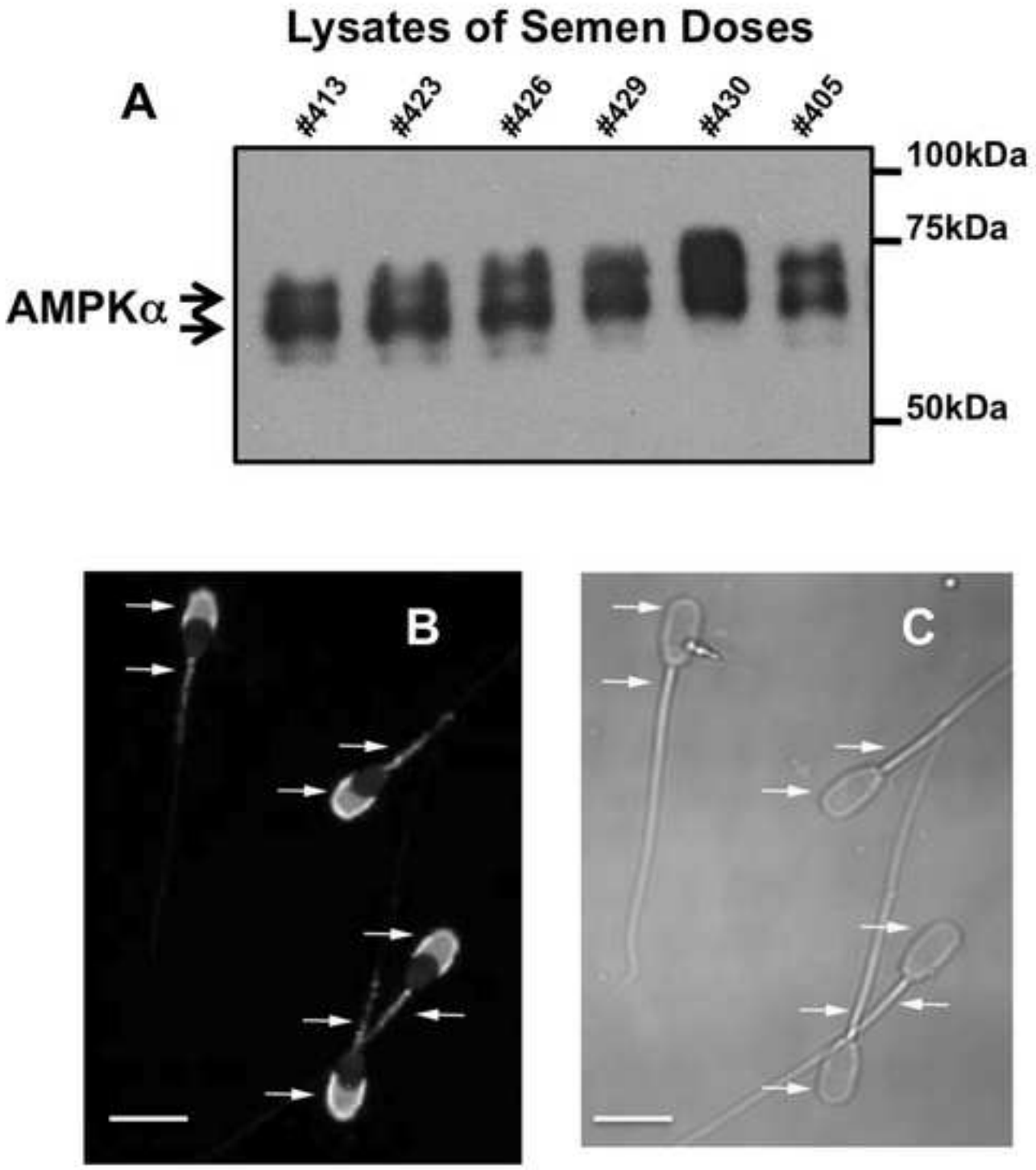

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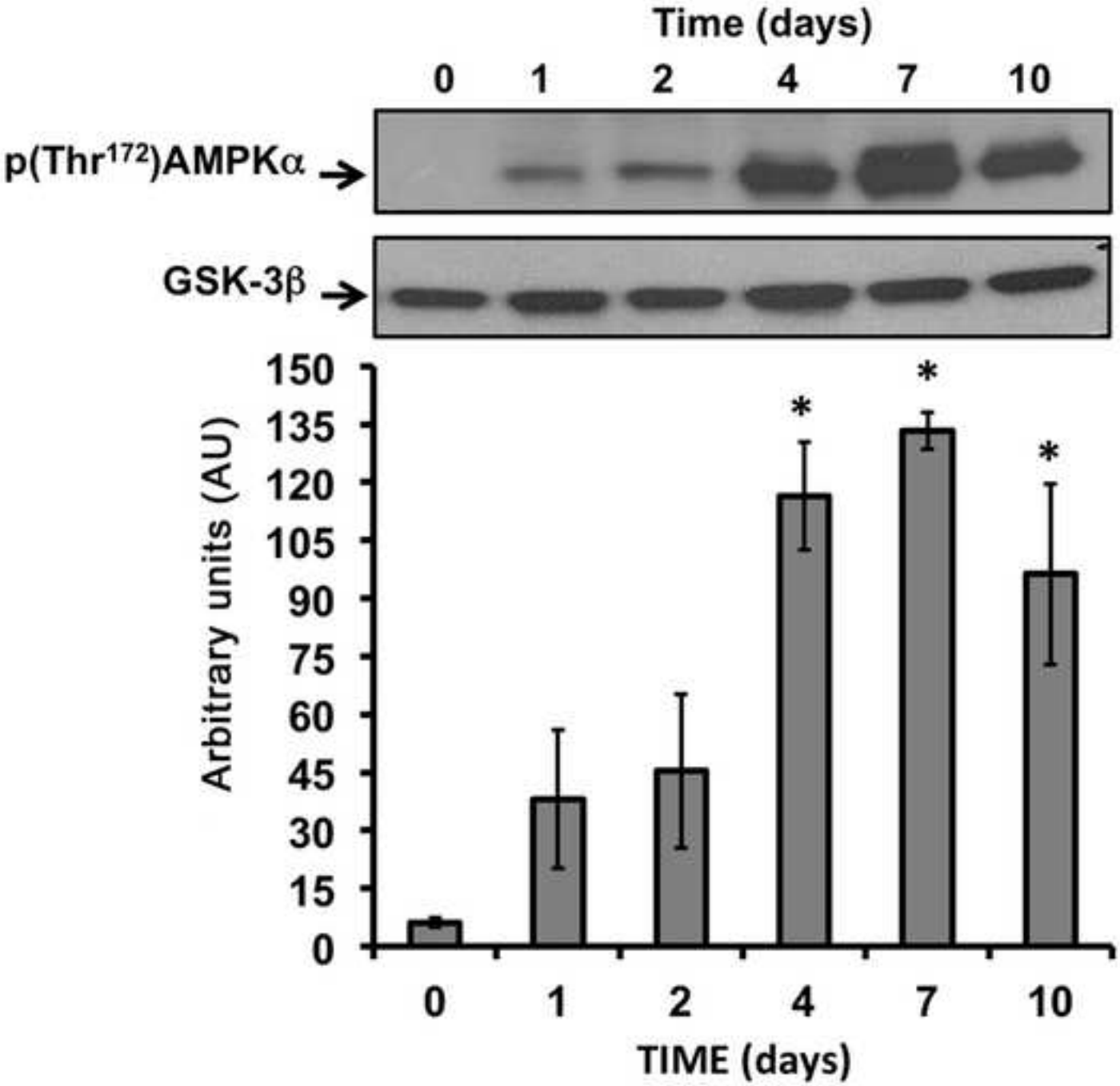

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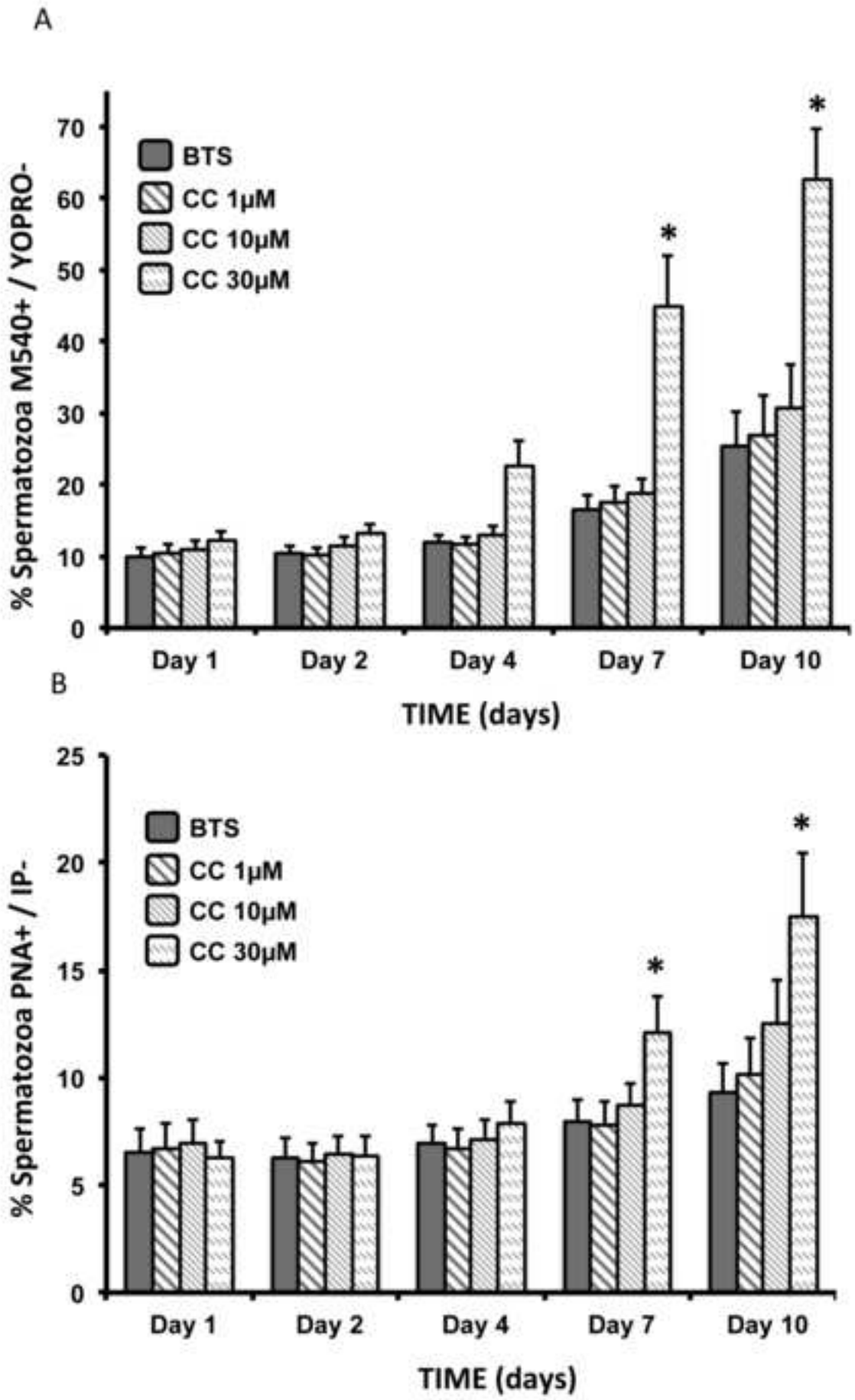

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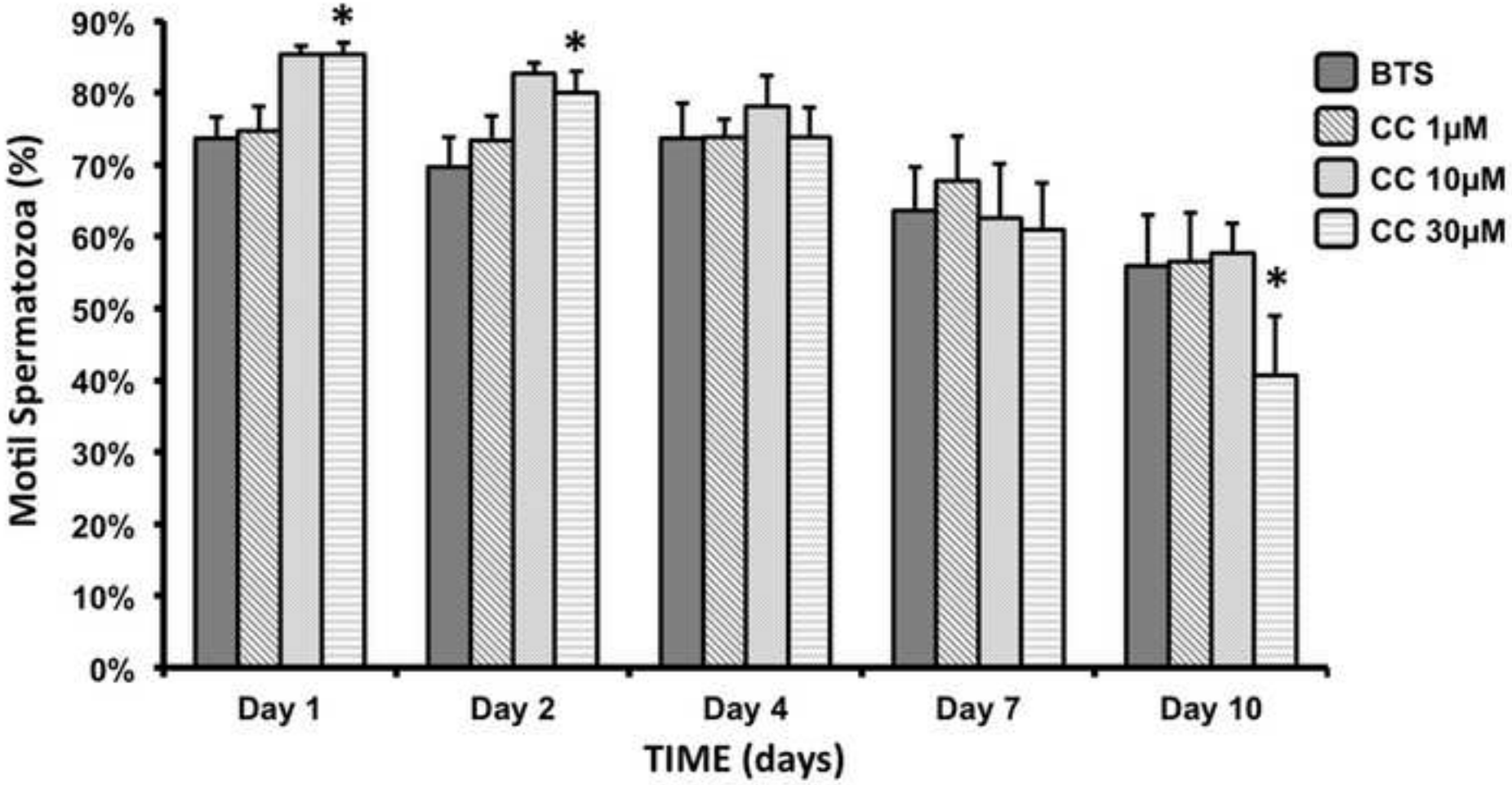

Figure 5

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