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Extracellular cAMP and MRP4 activity influence in vitro capacitation and fertilizing ability of pig spermatozoa

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ABSTRACT

cAMP has been reported to be an essential driver of sperm capacitation. In bovine sperm cAMP efflux through multidrug resistance-associated protein 4 (MRP4) has been suggested to maintain intracellular cAMP homeostasis and generate extracellular signaling able to regulate capacitation. The aim of this work was to determine whether extracellular cAMP may influence in vitro pig sperm capacitation and acquisition of fertilizing ability and to evaluate the role of MRP4. In vitro sperm capacitation and gamete coincubation were performed in Brackett and Oliphant's medium (BO) in presence of caffeine (Ctr+) or in BO without caffeine (Ctr-) supplemented with 0, 8, 9, 10 mM cAMP. Despite the percentage of capacitated sperm, assayed by immunolocalization of tyrosine-phosphorylated proteins, was significantly lower in Ctr- compared to Ctr+, it increased supplementing 10 mM cAMP to Ctr- reaching values similar to Ctr+. The absence of caffeine during gamete coincubation reduced the fertilization rate compared to Ctr+, while 10 mM cAMP supplementation to Ctr- increased the fertilization rate reaching values similar to Ctr + .

The presence of MRP4 in pig spermatozoa was detected for the first time by western blot and immunohistochemistry assays. To evaluate MRP4 role on pig sperm capacitation, in vitro capacitation and gamete coincubation were performed in Ctr + in presence of MK571, a MRP4 selective inhibitor. MK571 reduced the percentage of capacitated cells and the fertilization rate, while cAMP addition fully reversed MRP4 blockade consequences.

Present findings suggest that, under our in vitro conditions, extracellular cAMP and MRP4 activity influence pig sperm capacitating events.

1. Introduction

Mammalian ejaculated spermatozoa are morphologically mature however they are not still able to fertilize the mature oocyte as they need to undergo capacitation, fully acquiring fertilizing ability, during their residence in the oviductal sperm reservoir. Various physiological and biochemical changes occur during sperm capacitation including, but not limited to, the increase of cyclic AMP (cAMP) levels, influx of Ca2+ ions, generation of reactive oxygen species (ROS), cholesterol efflux from the plasma membrane leading to an increase in its fluidity, increase of intracellular pH, and activation of protein kinases resulting in protein phosphorylation of numerous proteins on tyrosine residues among others (Yanagimachi, 1994; Visconti et al., 2002; de Lamirande and O'Flaherty, 2008).

Cyclic AMP has been reported to be essential for events occurring during sperm capacitation (Buffone et al., 2014). After boar spermatozoa are exposed to factors (especially to bicarbonate) in the female reproductive tracts or culture media in vitro, the sperm-specific form of adenylyl cyclase (Okamura et al., 1985; Chen et al., 2000) is stimulated to increase intracellular levels of cyclic AMP that lead to protein kinase A activation (Gadella and Harrison, 2002; Harrison, 2004; Harayama, 2013; Funahashi, 2015a). Levels of intracellular cAMP are highly dynamic and depend on the concerted action of both synthesis and degradation. The cAMP inside the cell is degraded by phosphodiesterase (PDE), however it has been suggested that intracellular cAMP levels are also regulated by its efflux to the extracellular space. The extrusion of cAMP through multidrug resistance-associated protein 4 (MRP4), the main transporter associated with cAMP efflux, has been described in mouse and bull spermatozoa during in vitro capacitation (Osycka-Salut et al., 2014; Alonso et al., 2019). Pharmacological inhibition of MRP4

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blocked sperm cAMP efflux and, at the same time, prevented sperm capacitation suggesting an involvement of cAMP efflux via MRP4 in the modulation of capacitation regulating intracellular cAMP levels (Osycka-Salut et al., 2014; Alonso et al., 2019; Chiarante et al., 2020).

While in murine spermatozoa the role of cAMP extruded in the extracellular space has not been clarified (Alonso et al., 2019), in bovine spermatozoa a dual role of cAMP efflux to the extracellular compartment through MRP4 has been suggested both in maintaining intracellular cAMP homeostasis and in generating extracellular signaling able to regulate capacitation (Osycka-Salut et al., 2014; Chiarante et al., 2020). Indeed, the nucleotide extruded in the extracellular compartment has been demonstrated to be able to regulate bicarbonate-induced bovine sperm capacitation by activating A1 adenosine receptors (Osycka-Salut et al., 2014).

On these bases, the aim of this work was to determine whether extracellular non-permeable cAMP plays a role in the induction of capacitation and the acquisition of fertilizing ability of pig spermatozoa and to evaluate the role of MRP4 in this species using a well-defined in vitro model of pig sperm capacitation and fertilization (Spinaci et al., 2008; Spinaci et al., 2013; Bucci et al., 2012; Gadani et al., 2017; Spinaci et al., 2017; Spinaci et al., 2018).

2. Materials and methods

Unless otherwise specified, all the reagents were purchased from Sigma-Aldrich (Milan, Italy).

2.1. Semen collection

Sperm-rich fraction of ejaculates was collected by gloved-hand technique from three mature boars (Large White × Landrace × Duroc) of proven fertility aged between 2 and 3 years and extended in equal volume of Androhep TM (Minitub, Tiefenbach, Germany). Only ejaculates with sperm viability higher than 80% were used in the experiments. To balancing the sperm contribution of each male and eliminate variability between the evaluated semen samples, the sperm-rich fractions were pooled in this study.

2.2. In vitro sperm capacitation (IVC)

Aliquots of extended fresh semen were centrifuged at 900 x*g* for 2 min. After removal of supernatant, the pellet was resuspended with PBS supplemented with 0.4% (*w/v*) BSA and centrifuged again at the same condition. The final pellet was resuspended at the concentration of 30 × 10^6 with Brackett and Oliphant's medium (BO) (112 mM NaCl, 4.02 mM KCl, 2.25 mM $CaCl_2x2H_2O$, 0.83 mM $NaH_2PO_4xH_2O$, 0.52 mM $MgCl_2x6H_2O$, 37.0 mM $NaHCO_3$, 13.9 mM glucose, 1.25 mM sodium pyruvate) (Brackett and Oliphant, 1975) supplemented with 12% (*v/v*) fetal calf serum (FCS) (Gibco, Invitrogen, Italy) in absence (Ctr-) or presence of 0.7 mg/ml caffeine (Ctr+). Sperm cells were incubated in Nunc 4-well multidish for 1 h at 39°C in a humidified atmosphere of 5% CO_2 in air. At the end of incubation period, spermatozoa were subjected to the evaluation of viability and localization of tyrosine-phosphorylated proteins.

2.3. Evaluation of plasma membrane integrity

Twenty five microliters of semen from the different experimental groups (containing around 0,75 × 10^6 spermatozoa) were incubated with 2 µl of a 300 µM propidium iodide (PI) stock solution and 2 µl of a 10 µM SYBR green-14 stock solution, both obtained from the live/dead sperm viability kit (Molecular Probes, Invitrogen, Milan, Italy) for 5 min at 37°C in the darkness. At least 200 spermatozoa per sample were scored with a Nikon Eclipse E 600 epifluorescence microscope (Nikon Europe BV, Badhoeverdop, The Netherlands) by the same observer, blinded to the experimental group of each sample. Spermatozoa stained with SYBR green-14 and not stained with PI were considered as viable. Spermatozoa SYBR and PI positive and those SYBR negative / PI positive were considered as cells with compromised membrane or dead.

2.4. Immunofluorescence localization of tyrosine-phosphorylated proteins

All the procedures were carried out at room temperature unless otherwise specified.

Aliquots of sperm cells from the different experimental groups were deposited onto poly-L-lysine-coated slides and fixed with methanol at −20°C for 15 min and with acetone for 30 s. The slides were then washed with PBS and blocked with 10% (*v/v*) FCS (Gibco) in PBS (blocking solution) for at least 30 min. Antibody dilutions were performed in blocking solution. Monoclonal antiphosphotyrosine antibody (clone 4G10, Merck Millipore, Darmstadt, Germany) was added at the dilution 1:200. Incubation was carried out overnight at 4°C. After extensive washing with PBS, sperm cells were incubated with a sheep-anti-mouse FITC-conjugated secondary antibody (BioFX Laboratories, Maryland, USA) for 1 h in the dark at room temperature. Slides were washed with PBS and mounted with Vectashield mounting medium with PI (Vector Laboratories). Control slides were treated similarly with the omission of primary antiserum. Spermatozoa were evaluated with the above described epifluorescence microscope.

Each sample was analyzed by counting at least 200 cells in order to evaluate the different positivity patterns by the same observer, blinded to the experimental group of each sample.

Four different patterns were considered on the basis of what assessed by Bucci et al. (Bucci et al., 2012): A: positivity in the acrosome and Equatorial Subsegment (EqSS); B: positivity in the acrosome, EqSS and principal piece of the tail; C: positivity in the tail and (not constant) in the EqSS; NEG: spermatozoa with no positive signal.

2.5. Detection of MRP4 by western blot and immunocytochemistry

For MRP4 Western blot analysis, 10 × 10^6 spermatozoa were washed with PBS and centrifuged at 2000 ×*g* for 4 min. The pellet was then resuspended in 50 µL of RIPA Lysis Extraction Buffer (89,901 Thermo Fisher Scientific Rockford, IL, USA), then sonicated for 10 s and finally stored at −80°C until analysis. Total protein from 1 × 10^6 were separated on 4–12% bis-Tris Gel (Thermo Fisher Scientific Rockford, IL, USA) for 45 min at 165 V. Gels were washed in distilled water, pre equilibrated in 1× Tris-Glicine Buffer and then proteins were electrophoretically transferred onto a nitrocellulose membrane by Turbo Blot System (Bio-Rad Laboratories Inc., Hercules, CA, USA) as previously described (Bernardini et al., 2019).

Protein transfer was checked by staining both the nitrocellulose membranes with 0.2% Ponceau Red and the gels with Comassie Blue. Non-specific binding on nitrocellulose membranes was blocked with 5% (*w/v*) BSA in PBS-T20 (Phosphate Buffer Saline-0.1% Tween-20) for 1 h at room temperature. Then, the membranes were probed for the anti-MRP4 antibody (1:500 monoclonal anti MRP4 antibody, 12,857 Cell Signaling Technology, MA, USA) and the anti-β-tubulin antibody (1:500 of anti β-tubulin MA1–19162, Thermo Fisher Scientific, Rockford, IL, USA). After several washings with PBS-T20, the membranes were incubated at first with an appropriate dilution of secondary biotinylated antibody and then with a 1:1000 dilution of antibiotin antibody horseradish peroxidase linked. Finally, the membranes were developed using a chemiluminescent kit according to the manufacturer's instructions (Clarity Biorad Laboratories Inc., Hercules, CA, USA). The intensity of the luminescent signal of the resultant specific bands was acquired by Chemidoc Instrument using Image Lab Software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

For immunocytochemistry, aliquots of extended fresh semen were fixed in 0.2% *w/v* paraformaldehyde for 20 min at room temperature. Cells were immobilized on poly-L-lysine-coated slides and permeabilized with 0.5% (*v/v*) Triton X100 in PBS for 20 min at room temperature.

Non-specific binding sites were blocked with 3% (*w/v*) BSA in PBS for 1 h and then slides were incubated with primary MRP4 antibody (1:50) overnight at 4°C in blocking solution. After extensive washing with PBS, slides were incubated with an anti-rabbit FITC-conjugated secondary antibody for 1 h in the dark at room temperature. Specificity of the immunodetection was assessed by omitting the first antibody. Slides were washed with PBS and mounted with Vectashield mounting medium with PI (Vector Laboratories). Spermatozoa were evaluated with the above described epifluorescence microscope.

2.6. In vitro fertilization assay

2.6.1. Oocytes collection and in vitro maturation (IVM)

Ovaries were collected from pre-pubertal gilts at a local slaughterhouse and transported (in 0.9% *w/v* NaCl solution) to the laboratory within 2 h. Cumulus-oocyte complexes (COCs) were aspirated from antral follicles, 3–6 mm in diameter, with a 18-gauge needle fixed to a 10-ml disposable syringe. Intact COCs were selected under a stereomicroscope and only COCs with more than three layers of compact cumulus cells and with uniform cytoplasm were transferred into a petri dish (35 mm, Nunclon, Denmark) prefilled with 2 ml of modified PBS supplemented with 0.4% BSA. After three washes in NCSU 37 (Petters and Wells, 1993) supplemented with 5 μg/ml insulin, 1 mM glutamine, 0.57 mM cysteine, 10 ng/ml epidermal growth factor (EGF), 50 μM β-mercaptoethanol and 10% porcine follicular fluid (IVM medium), groups of 50 COCs were transferred to a Nunc 4-well multidish containing 500 μL of the same medium per well and cultured at 39°C in a humidified atmosphere of 5% CO_2 in air. For the first 22 h of in vitro maturation the medium was supplemented with 1.0 mM db-cAMP and 0.12 IU/ml Pluset (Carlier, Italy). For the last 22 h COCs were transferred to fresh maturation medium (Funahashi et al., 1997).

2.6.2. In vitro fertilization (IVF)

At the end of the maturation period, 45 to 50 oocytes freed from cumulus cells were washed twice in Ctr + medium or Ctr – medium, transferred into each well of Nunc 4-well multidish containing 500 μl of the same medium and coincubated with 0.75–1 × 10^6 sperm/ml. After 1 h of gamete coincubation, oocytes were washed twice, transferred to fresh medium and cultured for 17 h until mounting on microscope slides and fixing in acetic acid/ethanol (1: 3) for 24 h. After staining with Lacmoid the oocytes were observed under a phase-contrast microscope. Fertilization rate (number of oocytes having a single female pronucleus and a single or multiple penetrated sperm nuclei or male pronuclei/total inseminated) and efficiency of IVF (number of oocyte containing a single female pronucleus and only one sperm head-male pronucleus/total inseminated) were recorded.

2.7. Experimental design

To evaluate whether extracellular cAMP is able to modulate in vitro-induced pig sperm capacitation, sperm cells were incubated as above described in Ctr + medium (supplemented with caffeine) or in Ctr – medium (without caffeine) supplemented with 0, 8, 9, 10 mM cAMP (4 replicates). In order to assess the role of extracellular cAMP on sperm fertilizing ability, sperm-oocyte coincubation was performed as above described in Ctr + medium or Ctr – medium supplemented with 0, 8, 9, 10 mM cAMP (5 replicates; total number of oocytes examined 1029).

A second set of experiments were performed in order to evaluate the role MRP4 (the main transporter associated with cAMP efflux) on pig sperm capacitation and fertilizing ability. To this aim, either in vitro of sperm capacitation (4 replicates) or gamete coincubation (5 replicates; total number of oocytes examined 620) were performed in Ctr + medium in presence of 0, 50, 100 μM MK571, a MRP4 selective inhibitor. Moreover, to evaluate if extracellular cAMP supplementation is able to rescue the effect on pig sperm due to MRP4 blockade, in vitro sperm capacitation (4 replicates) or gamete coincubation (5 replicates; total number of oocytes examined 568) were performed in Ctr + medium alone or in presence of either 50 μM MK571 or 50 μM MK571 in combination with 10 mM cAMP.

2.8. Statistical analysis

Data were analyzed using R (version 4.2.1) (R Core Team, 2017). Values are expressed as mean ± standard deviation (SD) and level of significance was at $p < 0.05$.

Shapiro Wilk test was applied to test normality and Levene test was used to assay homogeneity of the variances. One-way ANOVA and Tukey post hoc test were performed to assess the difference between treatments.

In vitro fertilization data were analyzed by a generalized linear model (GLM) and a Tukey post hoc test was used to assess difference between treatments.

3. Results

3.1. Effect of extracellular cAMP on spermatozoa during in vitro capacitation

Analysing tyrosine phosphorylation of proteins, when sperm cells were incubated for 1 h in absence of caffeine (Ctr-), a significantly ($p < 0.01$) higher percentage of cells displaying A pattern (non capacitated cells) and a lower percentage of cells displaying B pattern (capacitated cells) compared to Ctr + group was observed (Fig.1A). The supplementation of non-permeable cAMP to Ctr- medium induced a decrease of A pattern cells and a parallel increase of B pattern ones, significant ($p < 0.01$) at the higher concentration tested (10 mM). Moreover, cAMP supplementation to Ctr- medium at the concentration of 8 mM was able to increase the percentage of B pattern cells to values similar to those observed in presence of caffeine (Ctr + group) (Fig. 1A).

Sperm viability was significantly higher ($p < 0.05$) in Ctr- group compared to Ctr + group, while in presence of all the cAMP concentration tested the percentage of live sperm was similar to Ctr + group (Fig. 1B).

3.2. Effect of extracellular cAMP on IVF outcomes

When gamete coincubation was performed in absence of caffeine (Ctr-) both the penetration rate and the IVF efficiency dramatically dropped ($p < 0.001$) compared to Ctr + (Fig.2). The supplementation of non-permeable cAMP to Ctr- medium was effective in increasing the penetration rate compared to Ctr- group starting from the concentration of 8 mM ($p < 0.05$), reaching values similar to Ctr + group at the concentration of 10 mM (Fig.2). Similarly, the IVF efficiency progressively increased in Ctr- group when cAMP was added, reaching Ctr + levels at the concentration of 9 mM.

3.3. Detection of MRP4 by western blot and immunocytochemistry

Fig. 3A shows a representative Western Blot of MRP4. A single immunoreactive band of the expected molecular weight was detected.

Immunofluorescence assay revealed a positive immunolabelling in the post-acrosomal region and in the anterior boundary of the equatorial segment of the sperm head (Fig. 3B).

3.4. Effect of MRP4 inhibition on in vitro-induced pig sperm capacitation and IVF

In order to evaluate the role of MRP4, the main transporter associated with cAMP efflux, on pig sperm capacitation, sperm cells were incubated in Ctr + medium for 1 h in presence of MK571, a MRP4 selective inhibitor. The addition of MK571 during in vitro capacitation at the concentrations tested (50 and 100 μM) progressively increased the

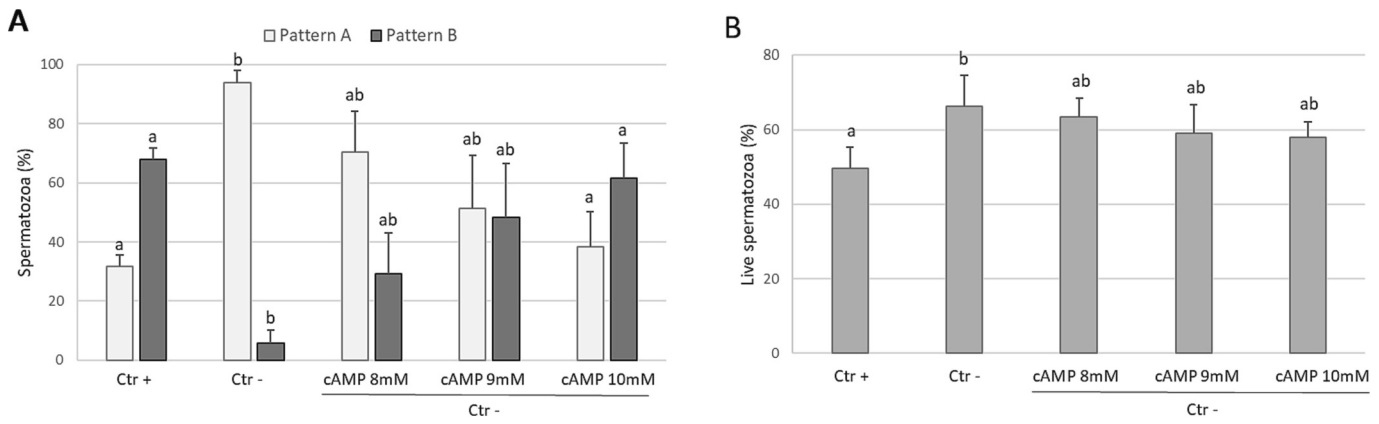

Fig. 1. Effect of supplementation of Ctr- medium with non-permeable cAMP at concentrations of 0, 8, 9, 10 mM during in vitro induction of sperm capacitation. Ctr +: BO medium supplemented with caffeine; Ctr-: BO medium without caffeine. (A) percentage of sperm cells displaying tyrosine-phosphorylation of sperm proteins typical of either non capacitated cells (pattern A) or capacitated cells (pattern B); (B) Sperm viability. Data represent the mean ± SD of 4 replicates repeated in different experiments. Different letters on the same bar type represent significant difference for $p < 0.05$ between treatments.

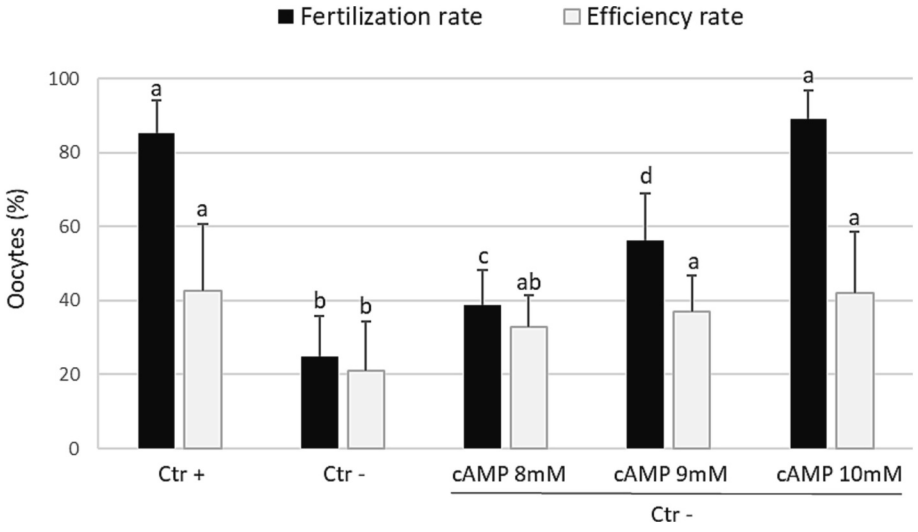

Fig. 2. Effect of supplementation of Ctr- medium with non-permeable cAMP at concentrations of 0, 8, 9, 10 mM during sperm-oocyte coincubation on IVF parameters. Ctr +: BO medium supplemented with caffeine; Ctr-: BO medium without caffeine. Fertilization rate: number of oocytes fertilized/total inseminated; Efficiency rate: number of oocyte containing only one sperm head-male pronucleus/total inseminated. Data represent the mean ± SD of 5 replicates repeated in different experiments. Different letters on the same bar type represent significant difference for $p < 0.05$ between treatments.

percentage of cells displaying A pattern (50 μM, $p < 0,05$; 100 μM, $p < 0.01$) and reduced the that of B pattern cells ($p \langle 0,01$) (Fig. 4A).

Sperm viability was not influenced by MK571 addition confirming the absence of a cytotoxic effect of the inhibitor (36.4 ± 1.7% vs. 42.7 ± 14.5% vs. 45.5 ± 16.3%, Ctr + vs. MK571 50 μM vs. MK571 100 μM).

When gamete coincubation was performed in presence of MK571 the fertilization rate and the IVF efficiency were negatively influenced at both the concentration tested ($p < 0,01$) (Fig. 4B).

3.5. Effect of simultaneous supplementation of MK571 and cAMP on sperm protein tyrosine phosphorylation and IVF outcomes

When MRP4 was inhibited by MK571 during IVC, the decrease of the percentage of sperm cells displaying B pattern ($p < 0,05$) was completely restored up to Ctr + levels by the addition of extracellular cAMP (Fig. 5A). Moreover, when gamete coincubation was performed in presence of MK571, cAMP supplementation reversed the inhibitory effect induced by MRP4 blockade leading to fertilization and efficiency rate higher compared to Ctr + group ($p < 0.01$) (Fig .5B).

4. Discussion

The primary findings of the present study are: i) The addition of extracellular non-permeable cAMP stimulates in vitro pig sperm capacitation and fertilizing ability; ii) MRP4, the main transporter associated with cAMP efflux, is present in boar spermatozoa; iii) MRP4 inhibition reduces pig sperm capacitation and fertilizing ability; iiii) The addition of extracellular cAMP fully reverses MRP4 blockade consequences.

As a first step of this study, we investigated if extracellular cAMP is involved in the modulation of pig sperm capacitation under our in vitro conditions of pig sperm capacitation and fertilization (Spinaci et al., 2008; Spinaci et al., 2013; Bucci et al., 2012; Gadani et al., 2017; Spinaci et al., 2017; Spinaci et al., 2018). Spermatozoa were incubated in BO medium supplemented or not with caffeine, a cyclic nucleotide phosphodiesterase inhibitor, whose activity results in an increase in intracellular cAMP levels and, for this reason, is supplemented in most porcine IVF systems to sufficiently activate capacitating signal transduction in pig spermatozoa (Harayama, 2013; Funahashi, 2015b). Tyrosine phosphorylation of sperm proteins is a process highly related

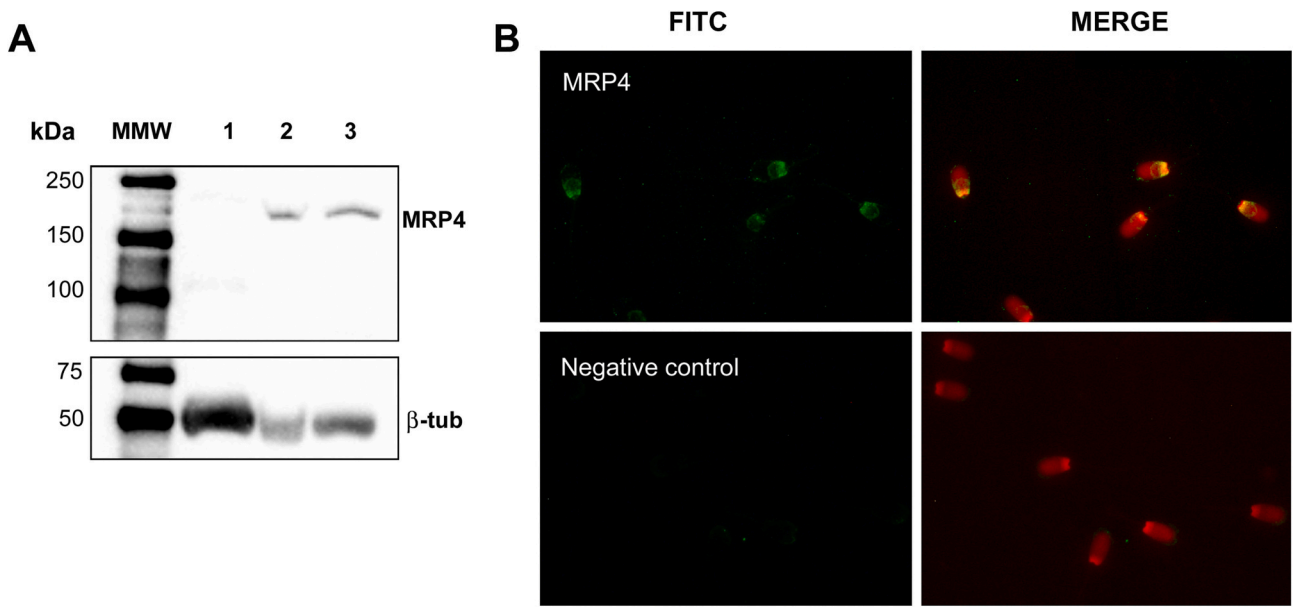

Fig. 3. Expression and localization of MRP4 in pig spermatozoa.
(A) Representative Western blot of MRP4 and β-tubuline. Lane 1: 1×10^6 pig normal fibroblasts (negative control); lane 2: 1×10^6 pig spermatozoa; lane 3: 1×10^6 HT-29 cells (positive control).
(B) Representative fluorescent micrographs of MRP4 immunolocalization on boar spermatozoa. Green fluorescence indicates positive MRP4 immunostaining revealed by FITC-conjugated secondary antibody. In the right panels (merge), red fluorescence indicates nuclear staining with PI and yellowish staining indicates the overlap of FITC and PI staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

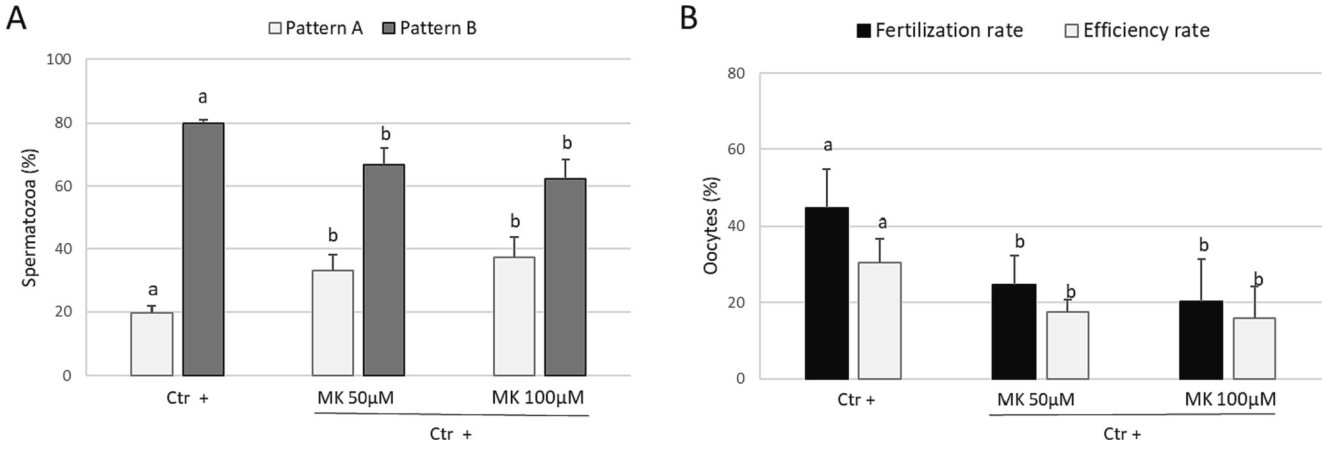

Fig. 4. Effect of MK571 (MK), a selective MRP4 inhibitor, on sperm capacitation and IVF parameters. Ctr +: BO medium supplemented with caffeine. (A) percentage of sperm cells displaying tyrosine-phosphorylation of sperm proteins typical of either non capacitated cells (pattern A) or capacitated cells (pattern B); (B) IVF parameters: Fertilization rate (number of oocytes fertilized/total inseminated) and efficiency rate (number of oocyte containing only one sperm head-male pronucleus/total inseminated). Data represent the mean ± SD of 4 replicates for immunolocalization of sperm phosphotyrosine and 5 replicates for IVF repeated in different experiments. Different letters on the same bar type represent significant difference for $p < 0.05$ between treatments.

with the capacitation process and is modulated through cAMP-dependent pathways (Visconti et al., 1999). As expected, the absence of caffeine (Ctr-) in BO medium during sperm IVC reduced the abundance of sperm population displaying a B pattern of phosphotyrosine immunopositivity (characteristic of capacitated cells) (Bucci et al., 2012). When BO medium without caffeine (Ctr-) was supplemented with non-permeable cAMP, an increase in the percentage of B pattern spermatozoa was recorded, percentage that, in presence of extracellular cAMP at the concentration of 10 mM, reached similar levels to those observed in presence of caffeine (Ctr+). These results suggest that in pig extracellular cAMP is involved in the process of in vitro-inducted sperm capacitation exerting a stimulatory effect on sperm population. This finding agrees with those obtained in bovine spermatozoa in which the extracellular nucleotide has been observed to induce a significant increase in sperm tyrosine phosphorylation, as evaluated by western blot, when compared to the control. In this species, an increase in capacitation associated events induced by extracellular cAMP was also demonstrated by CTC assay and LPC-induced acrosome reaction (Osycka-Salut et al., 2014). Furthermore, incubation of bovine spermatozoa in presence of non-permeable cAMP has been shown to induce capacitation associated events such as sperm hyperactivation (Alonso et al., 2017) and increase actin polymerization (Chiarante et al., 2020). It has been suggested that the effect of extracellular cAMP on bovine sperm capacitation is exerted through the activation of A1 adenosine receptors presumably through the metabolization of cAMP to adenosine by ecto-PDEs and ecto-nucleotidases (Chiavegatti et al., 2008) even if it cannot be excluded a direct effect of extracellular cAMP (Osycka-Salut et al., 2014). Alonso et al. (Alonso et al., 2017) elucidated the molecular

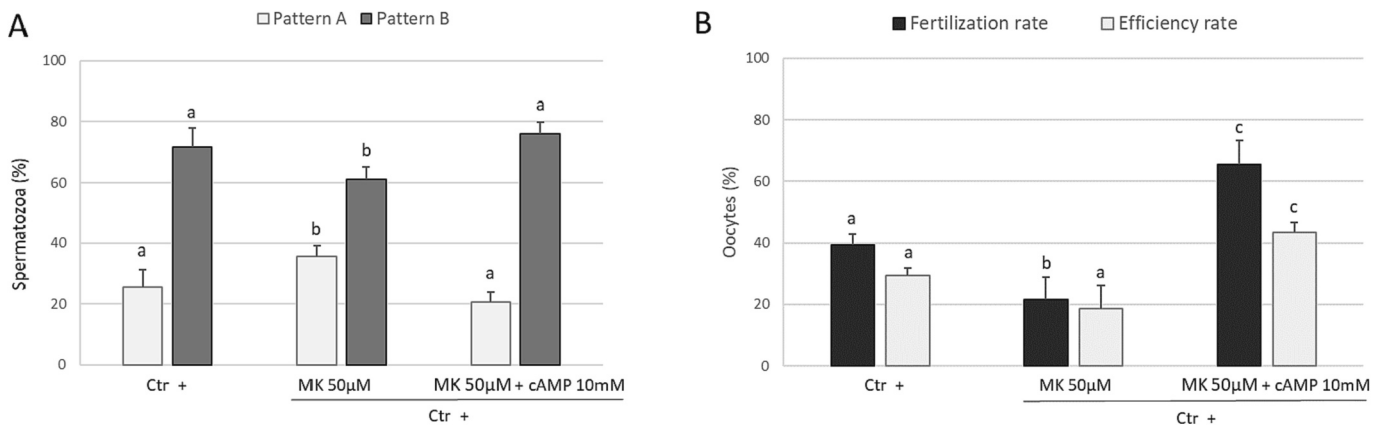

Fig. 5. Effect of MK571 (MK), a selective MRP4 inhibitor, and non-permeable cAMP on sperm capacitation and IVF parameters. Ctr +: BO medium supplemented with caffeine. (A) percentage of sperm cells displaying tyrosine-phosphorylation of sperm proteins typical of either non capacitated cells (pattern A) or capacitated cells (pattern B); (B) IVF parameters: Fertilization rate (number of oocytes fertilized/total inseminated) and efficiency rate (number of oocyte containing only one sperm head-male pronucleus/total inseminated). Data represent the mean ± SD of 4 replicates for immunolocalization of sperm phosphotyrosine and 5 replicates for IVF repeated in different experiments. Different letters on the same bar type represent significant difference for $p < 0.05$ between treatments.

events induced by extracellular cAMP in bovine sperm showing that A1 receptor stimulates phospholipase C that downstream increases ERK1–2 activation through PKC and induces a rise in sperm Ca2+ levels; furthermore, extracellular cAMP-induced capacitation is also depended on the activity of sAC and PKA. Further studies are required to elucidate the mechanism through which extracellular cAMP exerts the stimulatory effect on in vitro pig sperm capacitation observed in this study.

While the percentage of live cells after 1 h incubation was higher in absence of caffeine (Ctr-) compared to Ctr + group, the addition of cAMP to Ctr- medium slightly reduced sperm viability to values similar to Ctr + group. This observation further supports the hypothesis of a role of extracellular cAMP on pig sperm capacitation as a drop in sperm viability is normally associated with in vitro boar sperm capacitation process (Spinaci et al., 2018; Ramió-Lluch et al., 2014).

As capacitation is the process that enables spermatozoa to gain the ability to bind to the zona pellucida, penetrate it and finally fuse with the oocyte, in order to fully evaluate the biological effect of extracellular cAMP on boar sperm functions, gamete coincubation during IVF was performed either in medium supplemented with caffeine (Ctr+) or in medium void of caffeine (Ctr-) in presence of increasing concentrations of extracellular cAMP. According with the results obtained on capacitation parameters, cAMP supplementation to Ctr- medium progressively increased oocyte fertilization rate that, in 10 mM cAMP group, reached levels equal to those obtained when gamete coincubation was performed in presence of caffeine; these results confirm the powerful ability of the extracellular nucleotide to modulate in vitro pig sperm capacitation process. A similar stimulatory effect has been described in bovine by Alonso et al. (Alonso et al., 2017) who recorded analogous percentages of 2-pronuclei zygotes supplementing gamete coincubation medium with cAMP or heparin, a well-known stimulator of bovine sperm capacitation (Parrish, 2014).

The presence of MRP4 in pig spermatozoa was detected for the first time by western blot and immunohistochemistry assays. The localization of MRP4 in the post-acrosomal region and in the anterior boundary of the equatorial segment of the sperm head was similar to that recorded in bovine sperm (Osycka-Salut et al., 2014), even if no immunopositivity was recorded in sperm tail.

As already mentioned, it has been demonstrated that in mouse and bovine spermatozoa MRP4 extrudes cAMP to the extracellular space and that in bovine spermatozoa this event provides the nucleotide to the extracellular space for further signaling (Osycka-Salut et al., 2014; Chiarante et al., 2020). Thus, we investigated whether MRP4 activity may be involved in the modulation of in vitro capacitation of pig spermatozoa by incubating sperm cells in BO medium supplemented with caffeine (Ctr+) in presence of MK571, a selective inhibitor of MRP4. MK571 at both the concentrations tested significantly reduced the percentage of capacitated spermatozoa with tyrosine-phosphorylated protein positivity of tail principal piece (B pattern). Moreover, the presence of MK571 during gamete coincubation caused a drop in the percentage of fertilized oocytes suggesting that MRP4 activity plays a role in the stimulation of capacitation-associated acquisition of fertilizing ability of pig sperm population. Similar inhibitory effects of MRP4 blockade were obtained in bovine and mouse spermatozoa (Osycka-Salut et al., 2014; Alonso et al., 2019; Alonso et al., 2017). It could be hypothesized that also in pig sperm, as observed in mouse and bovine, the inhibitory effect of MRP4 blockade is linked to a inhibition of cAMP extrusion, however, further studies are required to support this hypothesis.

In our study the inhibitory effect exerted by MK571 on pig sperm capacitation and fertilizing ability was fully reversed by the addition of extracellular cAMP to the medium as it restored the proportion of sperm cells displaying a B pattern of protein tyrosine phosphorylation up to values observed in presence of caffeine (Ctr+) and improved the fertilization rate to levels higher than Ctr + group. These findings agree with those obtained in bovine spermatozoa (Osycka-Salut et al., 2014; Chiarante et al., 2020) but not with those recorded in mouse ones in which non permeable cAMP failed to overcome the consequence of MRP4 blockade (Alonso et al., 2019). Chiarante et al. (Chiarante et al., 2020) suggested that sperm MRP4 plays a species-specific role: in mouse the efflux of cAMP through MRP4 may only regulate cAMP intracellular levels, and in turn, the associated events, while in bovine sperm cAMP efflux by MRP4 provides the nucleotide in the extracellular environment where it can act in an autocrine/paracrine mode to stimulate sperm capacitation. Even though frozen-thawed bull spermatozoa were used in bovine studies, epididymal spermatozoa in the mouse one, and freshly ejaculated boar spermatozoa in the present study, it cannot be excluded that these differences may have had an influence on the results obtained. Nevertheless, our findings seem to confirm the hypothesis of Chiarante et al. (Chiarante et al., 2020) on sperm MRP4 species-specific role; indeed, porcine spermatozoa seem to behave in a similar way to bovine ones and in a different way than those of mice, regarding the ability of extracellular cAMP to overcome the inhibition of capacitation caused by MRP4 blockade.

In conclusion the present findings suggest that, under our in vitro conditions, extracellular cAMP plays a role in the modulation of some of the events associated with pig sperm capacitation and that MRP4 activity, possibly through cAMP extrusion to the extracellular space, may modulate capacitation in sperm population.

Authors contribution

MS designed the work. MS, OBP, JMOR, CB and DB conducted the experiments. MS wrote the manuscript. DB performed the statistical analysis. DB critically revised the work. All authors discussed the results and contributed to the final manuscript.

Author declaration

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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Prof. Marcella Spinaci.

CRediT authorship contribution statement

Marcella Spinaci: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft. **Olga Blanco-Prieto:** Investigation, Writing – review & editing. **Jose Manuel Ortiz-Rodriguez:** Investigation, Writing – review & editing. **Chiara Bernardini:** Investigation, Methodology, Data curation, Writing – review & editing. **Diego Bucci:** Data curation, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

None of the authors have conflict of interest to declare.

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