Reactive oxygen species are involved in the signaling of equine sperm chemotaxis

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Abstract

Sperm chemotaxis may facilitate the finding of the oocyte. Only capacitated spermatozoa can orient their movement by chemotaxis, which as well as capacitation, is regulated in part by the cAMP-PKA pathway. Reactive oxygen species (ROS) are produced during sperm capacitation which is closely related to chemotaxis. Then, the ROS participation in the chemotactic signaling can be expected. Here we studied the role of ROS in the chemotaxis signaling of equine spermatozoa which produce high quantities of ROS because of their energy metabolism. The level of capacitated and chemotactic spermatozoa was increased with 0.1 and 0.2 mM hydrogen peroxide (H₂O₂), which was involved in the chemotactic signaling. By combining a concentration gradient of H₂O₂ with inhibitors/chelators of some of the signaling pathway elements, we showed that the activation of NOX (membrane NADPH oxidase) increases the intracellular ROS which activate the chemotaxis AMPc-PKA pathway. Our results provide evidence about the participation of ROS in the chemotactic signaling mediated by progesterone (P).

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Introduction

Mammalian sperm capacitation is an essential process to guarantee fertilization of the mature oocyte (Austin 1951, Chang 1951). Early events during capacitation are the activation of the cAMP-PKA, of the increase of intracellular calcium (Ca²⁺), the production of reactive oxygen species (ROS), and activation of protein kinases, among others (Nakanishi *et al.* 2001, O'Flaherty *et al.* 2006b, Visconti *et al.* 2011, O'Flaherty 2015, Puga Molina *et al.* 2018). Once capacitated, sperm become capable of undergoing subsequent fertilization-related processes when exposed to the appropriate stimuli, including the chemotaxis response (Eisenbach & Giojalas 2006, Giojalas *et al.* 2015), the induced acrosome reaction (AR; Buffone 2016), the zona pellucida binding and penetration, and finally, the fusion with the plasma membrane of the female gamete (Florman & Fissore 2014).

It is well established that mammalian sperm capacitation is an oxidative event (Aitken *et al.* 1995,

de Lamirande *et al.* 1997, O'Flaherty 2015). Indeed, the early production of ROS evidenced by an increase in the concentrations of superoxide anion (O₂^{•-}), hydrogen peroxide (H₂O₂), nitric oxide (NO[•]), and peroxynitrite (ONOO⁻), has been reported during capacitation (de Lamirande & Gagnon 1995, Herrero *et al.* 2003, O'Flaherty *et al.* 2003, 2004). In the spermatozoon, the intracellular ROS are produced by numerous alternative sources including mitochondria, lipoxygenase as well as various oxidases (Bize *et al.* 1991, Griveau *et al.* 1994, Aitken *et al.* 1995, Baumber *et al.* 2003, O'Flaherty 2015, Aitken 2018). The role of ROS during capacitation involves the activation of molecules located on the plasma membrane and in other sperm compartments. For instance, O₂^{•-} and NO[•] would participate activating an AC, which is known to produce cAMP (Zhang & Zheng 1996, Herrero *et al.* 2000, O'Flaherty 2015), activating in turn a protein kinase A (PKA), essential step to support tyrosine phosphorylation associated with sperm capacitation in all species studied to date (Visconti *et al.* 1995a, Visconti *et al.* 1995b, Lewis & Aitken 2001,

Lefièvre *et al.* 2002, Ecroyd *et al.* 2003, O'Flaherty *et al.* 2004). In mammalian spermatozoa as well as in somatic cells, ROS are maintained at physiological levels by an antioxidant system that includes enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPXs), thioredoxins (TRXs), peroxiredoxins (PRDXs), and other molecules (Halliwell & Gutteridge 2015). When the antioxidant system is unbalanced, the excess of ROS becomes a harmful product for cell metabolism (Halliwell & Gutteridge 2015). The abnormal increase in intracellular ROS levels is termed oxidative stress and is the result of a disequilibrium in either an excessive production of ROS and/or a reduction in the antioxidant activity (Halliwell 2006, Halliwell & Gutteridge 2015). The oxidative damage affects all cell components, altering several sperm functions like motility, mitochondrial activity, capacitation and acrosome reaction, also causing DNA fragmentation (Jones & Mann 1977, Jones *et al.* 1979, Aitken *et al.* 1989, de Lamirande & Gagnon 1995, Sikka *et al.* 1995, de Lamirande *et al.* 1997, Griveau & Le Lannou 1997, Rivlin *et al.* 2004), which in turn leads to infertility (Aitken *et al.* 1989, Gagnon *et al.* 1991, Agarwal *et al.* 2006).

Sperm chemotaxis, a chemical guiding mechanism that may orient spermatozoa to the egg surface (Eisenbach & Giojalas 2006, Giojalas *et al.* 2015), has also been reported to be affected by the excess of ROS (Sánchez *et al.* 2010). Even though spermatozoa can be attracted by several molecules, progesterone becomes of physiological importance since it is secreted by the cells that surround the oocyte, forming a stable concentration gradient along the cumulus oophorus (Teves *et al.* 2006, Guidobaldi *et al.* 2008). Interestingly, P induces chemotaxis at very low concentrations (pM), in capacitated spermatozoa of several species (Teves *et al.* 2006, 2009, 2010, Blengini *et al.* 2011, Gatica *et al.* 2013, Ernesto *et al.* 2015, Franchi *et al.* 2016, Guidobaldi *et al.* 2017, Dominguez *et al.* 2018), and the chemotaxis signaling has been partially described for human spermatozoa (Teves *et al.* 2009, Giojalas *et al.* 2015).

Given that equine spermatozoa produce high levels of ROS because of their energy metabolism (Gibb *et al.* 2014) and that ROS are intermediaries in the signaling pathway of capacitation, process closely associated to chemotaxis, the aim of this work was to study whether there is a critical level of ROS that stimulates the capacitated-chemotactic state of stallion sperm, and whether ROS are involved in the chemotaxis signaling in this species.

Materials and methods

Chemicals and reagents

Unless otherwise specified, chemicals were purchased from Sigma-Aldrich. Mouse monoclonal anti-phosphotyrosine (anti-PY) antibody (clone 4G10, cat #05-321) was obtained

from EMD Millipore. Alexa Fluor 488-conjugated anti-mouse secondary antibody was purchased from Invitrogen (Thermo Fisher Scientific) and horseradish peroxidase (HRP)-conjugated anti mouse antibody was from Vector Lab. Inc. (Burlingame, CA, USA). The inhibitor and chelators used were PF431396 (Selleck Biochem), H89 dihydrochloride (Cayman Chemical), TAT-cAMP sponge was produced in the lab of Dr Claudia N. Tomes (IHEM, Argentina) (Lucchesi *et al.* 2016).

Sperm preparation and capacitation

Cryopreserved equine semen samples (200×10^6 sperm/mL, 0.5 mL per straw) of proven fertility from Polo Pony and Criollo breeds were donated by the Laboratorio de Producción Equina, Facultad de Agronomía y Veterinaria, Universidad Nacional de Río Cuarto (Córdoba, Argentina). For each set of experiments three ejaculates from at least three different stallions were included. Biggers, Whitten and Whittingham medium (BWW; Biggers *et al.* 1971) was used, supplemented with 5 U/mL penicillin, 5 mg/mL streptomycin, and 20 mM HEPES buffer (osmolarity of 300 mOsm/kg). This medium was used for non-capacitating assays (NonCAP), while for assays requiring capacitated spermatozoa, the medium (CAP) was supplemented with 3 mM pentoxifylline, 25 mM NaHCO₃ and 0.3% (w/v) of BSA. Semen samples were thawed at 38.5°C for 30 s, and seminal plasma and cryoprotectant were removed by the migration-sedimentation technique (Risopatrón *et al.* 1996), with culture medium (either NonCAP or CAP) for 45 min at 38.5°C, in an atmosphere of 5% CO₂ in air. In the case of CAP medium, this period was considered as capacitating time (Dominguez 2019).

Sperm parameters associated to capacitation

Protein tyrosine phosphorylation (PY) determined by immunocytochemistry

The occurrence of phosphorylation on tyrosine residues was determined by immunofluorescence according to González-Fernández *et al.* (2013). Sperm samples were fixed for 20 min in 2% (w/v) formaldehyde in PBS, washed by centrifugation, suspended in PBS, placed on a slide pretreated with poly-L-lysine and let dry in air. Spermatozoa were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 min, washed three times with PBS, incubated for 1 h in PBS containing 3% (w/v) BSA (blocking solution), and washed three times with PBS. Spermatozoa were incubated with or without (negative control) the anti-phosphotyrosine MAB (4G10 mAb) diluted 1:500 in PBS with 3% (w/v) BSA overnight at 4°C. After, washing with PBS, the cells were incubated with the secondary antibody, Alexa Fluor 488-conjugated goat anti-mouse IgG (1:500), for 1 h at room temperature in the dark, and then washed three times with PBS. A drop of a fluorescence enhancer was added (Vectashield, Vector, Burlingame, CA, USA) to improve and preserve cell fluorescence. At least 200 cells per treatment were observed at 1000× magnification under a fluorescence microscope (Olympus BX 50). Four staining patterns were observed as follows: only principal piece, both head and principal piece, only head and unlabeled. The first two patterns are associated with capacitation, while the other

two are not (Pommer *et al.* 2003, Matamoros-Volante *et al.* 2018). The percentage of spermatozoa showing the two capacitation patterns was recorded.

Protein tyrosine phosphorylation (PY) determined by Western blot

The occurrence of phosphorylation on tyrosine residues was also determined by Western blot according to Marín-Briggiler *et al.* (1999). To obtain the sperm protein extracts, sperm of each treatment were centrifuged for 5 min at 600 *g*, the sperm pellets were resuspended with Laemmli sample buffer, heated, centrifuged, and the supernatants were recovered. Samples were supplemented with 5% 2-mercaptoethanol, boiled for 10 min, stored at -20°C until used. The extract of sperm samples under different treatments were sent for WB analysis from CEBICEM to IBYME, Buenos Aires. Solubilized proteins (corresponding to 2×10^6 cells per lane) were subjected to SDS-PAGE in 10% polyacrylamide gels and Western immunoblotting, using anti-pY (1:1000), anti-tubulin (1:1500) and anti-mouse HRP-conjugated secondary antibody (1:2000). The reactive bands were detected by enhanced chemiluminescence (ECL kit, GE-Amersham) using standard procedures.

Induced acrosome reaction

The ability of spermatozoa to undergo the induced AR was determined as an indirect indicator of the level of capacitated spermatozoa (Jaiswal *et al.* 1998). The acrosome status was detected in alive spermatozoa using *Pisum sativum* agglutinin fluorescein staining (PSA-FITC; Zoppino *et al.* 2012, Bustos *et al.* 2014). Briefly, sperm suspensions were divided into two aliquots, which were incubated with or without 8 μM calcium ionophore A23187 and 10 $\mu\text{g}/\text{mL}$ PSA-FITC in culture medium, at 38.5°C for 30 min. Then, the sperm suspensions were fixed in 2% (w/v) formaldehyde in PBS for 20 min at room temperature, washed by centrifugation in distilled water, and the pellets were smeared on slides and allowed to air dry. The status of the acrosome was observed in at least 200 cells at 1000 $\times$ magnification under a fluorescence microscope (Olympus BX 50), where acrosome-reacted spermatozoa presented a green fluorescent acrosome, while the ones with intact acrosome were unlabeled. The net percentage of AR was determined as the difference in the AR percentage between induced and spontaneous acrosome-reacted spermatozoa.

Sperm chemotaxis

This parameter was evaluated by means of the Sperm Selection Assay (SSA) which recruits capacitated sperm by chemotaxis (Gatica *et al.* 2013). This assay was run under conditions defined for equine spermatozoa according to Dominguez (2019). Briefly, the device used in the SSA consists of two wells, in which the sperm suspension at $2 \times 10^6/\text{mL}$ is placed in one well (W1) and the attractant solution or culture medium as negative control in the other (W2). The two wells are connected by a tube, through which the attractant diffuses from W2 to W1, forming a concentration gradient which stimulates sperm chemotaxis. The SSA device was incubated

at 38.5°C , in an atmosphere of 5% CO_2 on air for 10 min. At the end of the assay, the net sperm accumulation in W2 was determined. This parameter was calculated as the difference in the percentage of spermatozoa recovered from W2 with or without attractants.

Sperm motility and other kinematic parameters

Sperm motility was determined by video microscopy and image analysis according to Giarretta *et al.* (2017) with brief modifications. A light microscope (Nikon Instruments Inc.) equipped with a 10 $\times$ negative phase contrast plan objective and a digital video camera Nikon CMOS USB 2.0 (Nikon Instruments Inc) was used. The videos were recorded for 5 s at a resolution of 800×600 and 30 frames/s using the NIS Elements Imaging Software 3.01 (Nikon Instruments Inc.), and then converted to avi format. Ten microliters of spermatozoa at a concentration of 10×10^6 sperm/mL were put on a slide previously warmed at 38.5°C , and covered with a $18 \times 18\text{-mm}^2$ coverslip. The slide was placed over a thermal plate (38.5°C), and 5 s records were taken from five different fields selected at random. The percentage of sperm motility and other kinematic parameters (curvilinear velocity; mean traverse velocity; straight-line velocity) were determined by CASA-bmg plugin (Giarretta *et al.* 2017), using ImageJ software (version 1.46j; NIH). Only samples exhibiting $\geq 90\%$ of motile spermatozoa after sperm separation were included in the assays.

Flow cytometry

Unless otherwise stated, flow cytometry was performed using an Acuri C6 flow cytometer (Becton Dickinson) with a 488-nm argon ion laser. Emission measurements were made using 530/30 nm band pass (green/FL-1), 585/42 nm band pass (red/FL-2), 661/16 nm band pass (red/FL-3), and 670 nm long pass (far red/FL-4) filters. Debris was gated out using a forward scatter/side scatter dot plot, and 100,000 cells were analyzed per sample. All data were analyzed using FlowJo software v10.0.7 (Becton Dickinson).

Viability

Sperm viability was assessed using the LIVE/DEAD™ Viability/Cytotoxicity Kit (Invitrogen). Briefly, spermatozoa were incubated at 38.5°C for 20 min with 0.5 μM stain solution, according to the manufacturer's instructions. Sperm samples were analyzed by flow cytometry and classified as either viable or not viable, using a snap-frozen ('dead') positive control for gating purposes. Results were expressed as percentage of living cells.

ROS production

Cytoplasmic and mitochondrial superoxide anion production was measured according to Gibb *et al.* (2014). Briefly, spermatozoa were incubated with 2 μM dihydroethidium (DHE; Molecular Probes) and 0.5 μM Calcein-AM viability stain (Molecular Probes), for 15 min at 38.5°C . Samples were assessed by flow cytometry gating the alive sperm population

(positive for Calcein-AM) and positive for DHE (evidencing cytoplasmic ROS). Mitochondrial superoxide production was measured by incubating spermatozoa with 2 μ M MitoSOX Red (MSR; Molecular Probes) and 0.5 μ M Calcein-AM (Molecular Probes) for 15 min at 38.5°C. Samples were assessed by flow cytometry gating the alive sperm population (positive for Calcein-AM and for MitoSOX evidencing mitochondrial ROS). As a positive control for both MSR and DHE assays, 100 nM phorbol 12-myristate 13-acetate (PMA) was used. Results were expressed as the mean fluorescence intensity (MFI).

Lipid peroxidation

Oxidative degradation of lipids was measured using the green fluorescence marker, BODIPY C11 kit (Molecular Probes Inc.), according to Aitken *et al.* (2007). In brief, 2×10^6 spermatozoa were incubated with BODIPY C11 (final concentration 5 μ M) during 30 min at 38.5°C and washed prior to flow cytometry analysis. As positive controls, aliquots of sperm suspensions were additionally treated with 2 mM of H_2O_2 to induce lipid peroxidation. The results were expressed as the percentage of cells exhibiting green fluorescent labeling.

Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was determined by flow cytometry using the JC-1 probe as previously described by Gravance *et al.* (2000). Aliquots with 2×10^6 sperm per ml were incubated with a 500 nM solution of JC-1 for 15 min at 38.5°C. As a negative control, a 2 mM solution of Carbonyl cyanide-*m*-chlorophenylhydrazone cyanide (CCCP) was used for 30 min at room temperature, which is an uncoupler of the oxidative phosphorylation.

Oxidative DNA damage

Oxidative guanine adducts (8-hydroxy-20-deoxyguanosine [8OHdG]) were immunolabeled and then analyzed by flow cytometry (Gibb *et al.* 2014). Aliquots with 2×10^6 sperm per mL were washed by centrifugation twice in PBS for 5 min at 600 *g*. Then, chromatin was relaxed to facilitate probe access by incubation with a 2 mM solution of dithiothreitol (DTT) for 15 min at 38.5°C. Spermatozoa were fixed and permeabilized with 4% formaldehyde in PBS and 0.05% Triton X-100 in PBS, respectively. The non-specific antigenic sites were blocked with a 5% goat serum solution in PBS/0.5% Triton X-100, for 2 h at room temperature. Subsequently, the cells were incubated with the monoclonal anti-8OHdG mouse antibody (1:500 in a solution of 1.5% goat serum in PBS/0.5% Triton X-100) overnight at 4°C. The cells were then washed with PBS for 5 min and centrifuged for 5 min at 2,300 *g*, followed by an incubation of 1 h with the FITC-conjugated anti-mouse secondary antibody (1:500 in a solution of 1.5% goat serum-PBS/0.5% Triton X-100). As positive controls, aliquots of sperm suspensions were additionally treated with 2 mM of H_2O_2 for 60 min to induce oxidative DNA damage. The cells were then centrifuged, resuspended in PBS, and analyzed. The results were expressed as the percentage of spermatozoa with green fluorescent labeling.

Statistical analysis

Data were expressed as the mean $\pm$ standard error (S.E.M.) of at least three independent experiments. Differences between treatments were determined by means of one-way ANOVA, where data were previously verified to accomplish the parametric assumptions of homogeneity of variances and normality using Anderson–Darling test. Differences between pair of means were determined with a *posteriori* Tukey test. Statistical analysis was performed with the GraphPad Prism 6.01, considering statistically significant differences at a level of confidence ≤ 0.05 .

Results

Endogenous production of ROS during sperm capacitation

In contrast to other mammalian species, equine spermatozoa use oxidative phosphorylation as the main energy source, generating considerable amounts of ROS in the mitochondria (Gibb & Aitken 2016). We determined the increase of endogenous $O_2^{\bullet-}$ in the cytoplasm and mitochondria before and after sperm incubation under NonCAP and CAP media (Fig. 1A and B). A significant increase in $O_2^{\bullet-}$ production was observed in mitochondria after 45 min of incubation under CAP condition as compared to time 0 and 45 min in NonCAP medium ($P=0.0006$; Fig. 1B). This result shows that, in equine spermatozoa, the level of $O_2^{\bullet-}$ increases as a result of sperm incubation under capacitating conditions.

Characterization of the effect of exogenous H_2O_2 on sperm capacitation and chemotaxis

In order to define the range of exogenous H_2O_2 necessary to stimulate equine sperm capacitation, spermatozoa were incubated under capacitating and non-capacitating conditions, in the absence or presence of different H_2O_2 concentrations. A mixture of antioxidants (CAT, catalase; SOD, superoxide dismutase) was used as negative control. At the end of the incubation time, sperm parameters associated with capacitation (% PY positive cells labeled in the principal piece and % AR_i), chemotaxis (% sperm accumulation in W2), vitality, motility and other kinematic parameters were evaluated in each treatment (Fig. 2A).

Under capacitating conditions, incubation with 0.1 and 0.2 mM H_2O_2 led to a significant increase in the % PY positive cells (Fig. 2B and C), % AR_i (Fig. 2F), and chemotaxis (Fig. 2G) as compared to the absence of H_2O_2 ($P=0.0003$, $P=0.0002$, $P=0.0002$, respectively). However, in the presence of 0.4 mM H_2O_2 a reduction in the values of all these parameters was observed (Fig. 2B, C, D, E, F and G). Similar results were obtained for PY levels assessed by Western blot (Fig. 2D). In general, when the cells were incubated under non-capacitating conditions, the H_2O_2 concentrations affected sperm physiological

Figure 1 Endogenous production of ROS during sperm capacitation. (A) Experimental design where downward arrowhead indicates measured parameter. (B) Gating strategy for data analysis, spermatozoa were selected on a forward and side scatter plot (left panel) and then only live spermatozoa (Calcein-AM positives; central panel) were further gated to determine DHE (upper right panel) or MitoSOX (lower right panel) mean fluorescence intensity. Sample data at time 0 (red) and that of unlabeled control (gray) are shown (C) Relative level of superoxide anion (MFI/MFI_{t0}) in cytoplasm (by DHE) and in mitochondria (by MitoSOX), at 0 min and 45 min of incubation under non capacitating (NonCAP in grey) or capacitating conditions (CAP in black). MFI, medium fluorescence intensity. The black dots represent individual experiments. The results are expressed normalized with respect to the control at t₀, as the mean $\pm$ s.e.m. of at least five experiments. ^aSignificant difference vs t₀ ($P < 0.05$).

parameters in a similar way than that of CAP medium, but with less magnitude. Sperm viability and kinematic parameters remained constant even at 0.4 mM H₂O₂ ($P=0.616$ and $P=0.9861$ respectively; Fig. 2H and I). In contrast, a slight decrease in the percentage of motile cells was observed with 0.2 mM H₂O₂ while a dramatic decrease in motility was observed with 0.4 mM H₂O₂ ($P < 0.0001$; Fig. 2H). Since under capacitation conditions there was a tendency of 0.2 mM H₂O₂ concentration to stimulate a higher effect on sperm function in comparison to 0.1 mM H₂O₂ (Fig. 2B, C, D, E, F and G), with no effect on viability or most sperm kinematic parameters (Fig. 2H and I), we used 0.2 mM H₂O₂ hereafter. Consistently, the antioxidants significantly decreased the values of the three sperm parameters to levels similar to the control (Fig. 2G). In summary, low levels of exogenous H₂O₂ stimulate sperm parameters associated with capacitation, while higher concentrations negatively affected those parameters and sperm motility.

Effects of exogenous H₂O₂ on the oxidative state of spermatozoa

The level of oxidative stress was evaluated in sperm incubated in the presence or absence of 0.2 mM H₂O₂ (concentration that stimulates capacitation), 0.4 mM H₂O₂

(concentration that inhibits capacitation), and 2 mM of H₂O₂ (positive control for oxidative stress; Barbonetti *et al.* 2016), determining the levels of cytoplasmic and mitochondrial ROS, lipid peroxidation, mitochondrial membrane potential ($\Delta\psi$ m) and DNA oxidation (Fig. 3A). When the cells were exposed to 0.2 and 0.4 mM H₂O₂, the level of cytoplasmic and mitochondrial O₂^{•-}, as well as DNA oxidation did not change compared to the negative control, but were significantly decreased in comparison to the positive control ($P=0.0139$, $P=0.0204$ and $P=0.0161$, respectively) (Fig. 3B, C and D). The mitochondrial membrane potential was constant at any H₂O₂ concentration, even with 2 mM (positive control); hence, in this set of experiments the mitochondrial uncoupler CCCP was used as a negative control ($P < 0.0001$; Fig. 3E). Lipid peroxidation was not affected under 0.2 mM of H₂O₂, but it was significantly increased under higher H₂O₂ concentrations ($P=0.0007$; Fig. 3F). These results show that the concentration of H₂O₂ that stimulates the sperm function associated to capacitation did not produce oxidative stress in spermatozoa.

ROS as mediators of the chemotaxis signaling

Since equine spermatozoa exposed to a chemotactic P gradient have significantly higher levels of mitochondrial

Figure 2 Induction of capacitation and chemotaxis by exogenous H_2O_2 in equine sperm. (A) Experimental design, upward arrowhead indicates treatment, downward arrowhead indicates measured parameter; (B) Representative microphotography of PY immunostaining (green) and nucleus (blue), empty arrowhead shows non labeling for PY and filled arrowhead shows positive labeling for PY; (C) Percentage of sperm with PY labeling in principal piece under CAP and NonCAP conditions; (D) PY patterns in sperm incubated under these conditions; (E) Percentage of sperm ARI. under CAP and NonCAP conditions; (F) Percentage of sperm accumulation in W2; (G) Effect of antioxidants (SOD + CAT, 1000 U/mL) on sperm parameters associated to capacitation. (H) Motility (dark grey) and viability (light grey) of sperm exposed to different concentrations of H_2O_2 ; (I) Sperm velocity under different concentrations of H_2O_2 . PY, protein tyrosine phosphorylation; ARI, induced acrosomal reaction; CAP, capacitating medium; NonCAP, no capacitating medium; VCL, curvilinear velocity (dark grey bars); VAP, mean trajectory velocity (light grey bars); VSL, straight line velocity (white bars). The black dots represent individual experiments. The results are presented as the mean $\pm$ S.E.M. of at least five experiments performed with ejaculates from three different horses. ^aSignificant difference vs without H_2O_2 or antioxidants, ^bSignificant differences vs 0.2 mM of H_2O_2 ($P < 0.05$).

and cytoplasmic ROS (Dominguez 2019), it would be possible that ROS may participate in the molecular mechanism of chemotaxis toward P. Our hypothesis is that by removing ROS by antioxidants chemotaxis would be inhibited. To test this hypothesis, after capacitation spermatozoa were incubated with different concentrations of antioxidants (a combination of CAT and SOD which scavenge ROS), and then exposed to a gradient of P in the sperm selection device, where

the % sperm accumulation in W2 and kinematic parameters were determined (Fig. 4A). The treatment with antioxidants significantly reduced the chemotactic response to P in a dose-dependent manner ($P = 0.0015$; Fig. 4B). Neither sperm velocity nor sperm motility were affected by any concentration of antioxidants ($P = 0.6494$; Fig. 4C). To evaluate whether H_2O_2 act as a second messenger during the chemotaxis response, spermatozoa were exposed to a concentration gradient

Figure 3 Oxidative stress parameters as a function of exogenous H_2O_2 concentration. (A) Experimental design where upward arrowhead indicates treatment applied, and downward arrowhead the measured parameters; (B) Cytoplasmic ROS levels; (C) Mitochondrial ROS levels; (D) % Sperm DNA oxidation; (E) % Sperm with high mitochondrial membrane potential (high $\Delta\psi_m$); (F) % Sperm lipid peroxidation. Gating strategy for data analysis are shown, spermatozoa were selected on a forward and side scatter plot (upper panels), and these cell populations were used to carry out the corresponding histogram for each studied parameter (D, E and F). CCCP, mitochondrial uncoupling. The black dots represent individual experiments. The results are presented as the mean $\pm$ S.E.M. of at least three experiments carried out with ejaculates from three different horses. ^aSignificant differences vs without H_2O_2 ($P < 0.05$).

generated by 0.2 mM of H_2O_2 (which is permeable to the plasma membrane) in the absence of P (the extracellular ligand that stimulates chemotaxis). Therefore, H_2O_2 would act as a second messenger, by inducing *per se* the orientation of the sperm movement toward W2. We and other laboratories have used this strategy to study the participation of second messengers on the chemotactic response (Gundersen & Barrett 1980, Teves *et al.* 2009). Capacitated spermatozoa were exposed in the SSA to a gradient generated by 10 pM of P (as a positive control) or by 0.2 mM of H_2O_2 (Fig. 4D). When spermatozoa were exposed to a gradient generated by 0.2 mM of H_2O_2 , the % of sperm accumulation in W2 was similar to that observed under a P gradient ($P = 0.1543$; Fig. 4E). We next performed control experiments to verify that sperm accumulation observed in W2 containing H_2O_2 was due to chemotaxis rather than to other kinematic processes (Giojalas *et al.* 2015, Fig. 4F). The % of sperm accumulated in W2 was close to zero in the absence of a H_2O_2 gradient, showing a negative value when sperm faced a descending H_2O_2 gradient, as compared to the ascending H_2O_2 gradient ($P < 0.0001$; Fig. 4G). These results are consistent with a sperm accumulation in W2 due only to chemotaxis and show that ROS may act as a second messenger in the molecular mechanism of chemotaxis toward P.

Sequence of chemotaxis intracellular events mediated by H_2O_2

Since ROS are known to participate in the activation of the AMPc-PKA capacitation signaling, where this pathway is also involved in the initiation of sperm chemotaxis, we next studied in which steps of this chemotactic pathway H_2O_2 were involved (Teves *et al.* 2009). After capacitation, spermatozoa were incubated with different concentrations of inhibitors or chelators, and then exposed to a concentration gradient generated by 0.2 mM of H_2O_2 in the SSA device, determining sperm accumulation in W2 and kinematic parameters. As a positive control, spermatozoa were exposed to a gradient generated by 10 pM of P (Fig. 5A). To explore whether H_2O_2 are involved before or after the activation of adenylate cyclase (AC), capacitated spermatozoa were incubated with different concentrations of a protein permeable to the cell membrane that scavenges cytoplasmic cAMP (TAT-cAMP sponge; Lucchesi *et al.* 2016). The treatment with TAT-cAMP sponge significantly decreased the chemotactic response of spermatozoa exposed to a concentration gradient of H_2O_2 in a dose-dependent manner ($P = 0.0008$), similarly to the response observed in the positive P control (Fig. 5B). Thus, the increase of H_2O_2 occurs prior to the activation of

Figure 4 H₂O₂ participates in the chemotaxis signalling pathway mediated by progesterone.

(A, D and F) Experimental design, upward arrowhead indicates treatment applied, downward arrowhead indicates measured parameter. (B) Percentage of sperm accumulation in W2 after being preincubated with different concentrations of antioxidants. (C) Sperm kinematic parameters after the antioxidant treatment. (E) Percentage of sperm accumulation in W2 after being exposed to a gradient generated by 10 pM of P or 0.2 mM of H₂O₂. (G) Percentage of sperm accumulation in W2 in presence of a descending gradient of H₂O₂ (i), an homogeneous concentration of H₂O₂ (ii), and an ascending gradient of H₂O₂ (iii); The SSA showing the experimental design for each treatment are shown in the left. P, progesterone; CM, culture medium; S, sperm; SOD, superoxide dismutase; CAT, catalase; VCL, curvilinear velocity; VAP, mean trajectory velocity; VSL, straight-line velocity. The black dots represent individual experiments. The results are presented as the mean $\pm$ S.E.M. of at least five experiments performed with ejaculates from three different horses. ^aSignificant differences vs control without antioxidants, ^bSignificant differences vs ascending gradient of H₂O₂, ^cSignificant differences vs homogeneous concentration of H₂O₂ ($P < 0.05$).

the AC. We next investigate whether the chemotactic signal stimulated by the H₂O₂ gradient was suppressed by inhibiting PKA. Capacitated spermatozoa were incubated with different concentrations of H89, a specific inhibitor of PKA (Lochner & Moolman 2006). Treatment with H89 significantly decreased the chemotactic response of spermatozoa exposed to a gradient generated by 0.2 mM of H₂O₂, in a dose-dependent manner ($P < 0.0001$); a similar result was observed in the corresponding P positive control (Fig. 5C). These results show that H₂O₂ may play a role prior to the activation of PKA. To evaluate whether H₂O₂ participates upstream protein tyrosine phosphorylation, capacitated spermatozoa were incubated with different doses of PF431396, a specific inhibitor for the tyrosine protein kinase 2 (PYK2) and tyrosine protein kinase FER (Alvau et al. 2016). Results presented in Fig. 5D show that the inhibitor significantly decreased the chemotactic response of sperm exposed to a gradient generated by 0.2 mM of H₂O₂, in a dose-dependent manner; similar results were observed in the corresponding P control ($P < 0.0001$). These results suggest that the increase in H₂O₂ occurs before the phosphorylation of proteins on tyrosine residues mediated by FER and PYK2 enzymes. Taking into account that the intracellular calcium increase is one of the events that occur during the chemotactic signal (Teves et al. 2009), we evaluated whether this

phenomenon occurs before or after the increase in intracellular H₂O₂. Capacitated sperm were incubated with different concentrations of the intracellular calcium chelator, BAPTA-AM, and then exposed to a gradient of H₂O₂, or P as a control. Treatment with BAPTA-AM significantly decreased the chemotactic response of spermatozoa exposed to a gradient generated by 0.2 mM of H₂O₂ in a dose-dependent manner, like the P control ($P < 0.0001$) (Fig. 5E). These results suggest that H₂O₂ play a role probably before the intracellular calcium increase.

Considering that there must be an enzyme that produces ROS immediately after binding P to its receptor, we next investigated whether NOX5 (an enzyme located in the sperm plasma membrane in equine spermatozoa (Sabeur & Ball 2007) that produce ROS in some mammalian spermatozoa (Sabeur & Ball 2007, Musset et al. 2012, Setyawan et al. 2016)), is involved in sperm chemotaxis mediated by P. Capacitated spermatozoa were incubated with different concentrations of DPI (a generic inhibitor of several flavoproteins; Li & Trush 1998), and then exposed to a gradient generated by H₂O₂ or P as a control (Fig. 6A). Treatment with DPI significantly decreased the chemotactic response of sperm exposed to a P gradient in a dose-dependent manner ($P < 0.0001$), but not to a H₂O₂ gradient ($P = 0.0656$; Fig. 6B). To evaluate

Figure 5 H₂O₂ is involved in the cAMP-PKA chemotaxis signaling by progesterone (P). (A) Experimental design, where upward arrowhead indicates treatment applied, downward arrowhead indicates measured parameter. Percentage of sperm accumulation in W2 after exposure to a gradient generated by 0.2 mM of H₂O₂ or 10 pM of P, previously incubated with different doses of: (B) TAT-cAMP sponge, a chelator of cAMP, (C) H89, an inhibitor of PKA, (D) PF431396, an inhibitor of protein tyrosine phosphorylation, and (E) BAPTA-AM, a chelator of the intracellular calcium. The black dots represent individual experiments. SSA, sperm selection assay; P, progesterone. The results are presented as the mean $\pm$ S.E.M. of at least three experiments with ejaculates from three different horses. *Significant difference vs without inhibitor ($P < 0.05$).

whether the H₂O₂ involved in chemotaxis is produced by NOX5 which is activated by calcium (Bánfi *et al.* 2004), capacitated spermatozoa were treated with or without DPI or BAPTA-AM and exposed to a P gradient, determining total O₂^{•-} production by DHE (Fig. 6C). ROS levels decreased in a dose dependent manner when spermatozoa were exposed to a P gradient in the presence of DPI ($P = 0.0004$; Fig. 6D and E) as well as under the presence of BAPTA-AM ($P = 0.0001$; Fig. 6F and G). These results suggest that NOX5 may be responsible for ROS production during the chemotaxis signaling. It is worth noting that the range of concentration of inhibitors and chelators used in this study (TAT-AMPC sponge: 0–100 nM; H89: 0–50 μM; PF431396: 0–10 μM; DPI: 0–1 μM; BAPTA-AM: 0–100 μM) did not affect the sperm kinematic parameters, meaning that only chemotaxis was interfered (Fig. 7).

Discussion

ROS have a dual effect on sperm. On one hand, they participate in the regulation of sperm capacitation, a required event for fertilization (Aitken *et al.* 1995, 1998, de Lamirande & Gagnon 1995, Lewis & Aitken 2001, Baumber *et al.* 2003, Ecroyd *et al.* 2003, Villegas *et al.* 2003, O'Flaherty *et al.* 2005, 2006a, Boerke *et al.* 2013). On the other hand, an excessive production of ROS leads

to oxidative stress negatively affecting sperm function (Aitken *et al.* 2012, Aitken 2017). The latter is due to the fact that once oxidative stress has begun, it increases over time, as a direct consequence of lipid peroxidation, which alters membrane-dependent sperm functions (e.g., motility), triggering a greater generation of ROS in mitochondria, leading to apoptosis (Moazamian *et al.* 2015). Thus, the intracellular amount of ROS may be a pivotal key for their dual effect on sperm physiology. In this work we show that the level of intracellular O₂^{•-} increases under capacitating conditions in equine sperm, in agreement with other reports in this and other species (Baumber *et al.* 2003, O'Flaherty *et al.* 2003, de Lamirande & Lamothe 2009, Donà *et al.* 2011).

An oxidase apparently exclusive of the spermatozoon, which is located mainly in the plasma membrane of several mammalian species, including equine, is responsible for the production of part of the ROS which are delivered to the extracellular space, to then return inside the cell; however, the participation of cytosolic oxidases cannot be ruled out (Bize *et al.* 1991, Griveau *et al.* 1994, Aitken *et al.* 1995, Baumber *et al.* 2003, O'Flaherty 2015). In equine sperm, it was observed that most endogenous ROS are produced by NADH dehydrogenase at complex 1 of the mitochondrial electron transport chain, because oxidative phosphorylation is the main source of energy in these cells (Gibb *et al.* 2014, Gibb & Aitken 2016).

Figure 6 H₂O₂ involved in the chemotactic signaling towards P could be produced by NOX5. (A) Experimental design for panel B, upward arrowhead indicates treatment applied, downward arrowhead indicates measured parameter, DPI an inhibitor of NOX5. (B) Percentage of sperm accumulation in W2 after exposure to a gradient generated by 0.2 mM of H₂O₂ or 10 pM of P, previously incubated with different doses of DPI. (C) Experimental design for panels (D, E, F and G), upward arrowhead indicates treatment applied, downward arrowhead indicates measured parameter. (D and F) Representative example of one experiment. (E and G) Endogenous production of ROS after exposure to a gradient generated by 10 pM of P, previously incubated with different doses of DPI or BAPTA-AM respectively. P, progesterone; SSA, sperm selection assay; MFI, mean fluorescence intensity. The black dots represent individual experiments. The results are presented as the mean $\pm$ S.E.M. of at least three experiments with ejaculates from three different horses. ^aSignificant difference vs without inhibitor. ^bSignificant differences vs without P gradient ($P < 0.05$).

In this study, the exogenous addition of H₂O₂, which is permeable to the plasma membrane, may also stimulate capacitation and other sperm functions. Thus, a range between 0.1 mM - 0.2 mM of H₂O₂ stimulate the occurrence of sperm parameters associated with capacitation, such as protein phosphorylation on tyrosine residues, the induction of the AR, and the chemotactic selection of equine spermatozoa which was shown for the first time by us, without causing oxidative stress. Moreover, this effect was reversed by the addition of antioxidants. On the contrary, higher doses of H₂O₂ (0.4 mM) significantly decrease the parameters associated with sperm capacitation, observing oxidative stress only in lipid peroxidation (Jones & Mann 1977, Jones *et al.* 1979, Aitken *et al.* 1989, de Lamirande *et al.* 1997). In this study we observed that 0.2 mM H₂O₂ stimulates sperm function in contrast to the results obtained by Morielli and O'Flaherty (2015) who observed a decrease in the induced AR when human spermatozoa were incubated with 0.25 mM H₂O₂. This apparent controversy may be due to several facts. Our study was performed in equine spermatozoa which energetic metabolism is different from that of human sperm; we used a lower concentration of H₂O₂ (0.2 mM

vs 0.25 mM) which stimulate sperm function without inducing oxidative stress, nor affecting cell viability and kinematic; even though in our study the percentage of motile cells decreased around 20% with 0.2 mM H₂O₂ (vs. 50% in their study) those moving spermatozoa show good kinematic parameters. Our findings are in agreement with previous studies which reported that similar concentrations of H₂O₂, stimulate PY in bovine, while higher concentrations of H₂O₂ are detrimental for spermatozoa (Rivlin *et al.* 2004). Interestingly, the induction of H₂O₂ production stimulates PY and the induced AR in equine spermatozoa (Baumber *et al.* 2003). Likewise, high levels of ROS produced by pharmacological induction, during a capacitating incubation for 4 h, inhibit the chemotactic response of human sperm to follicular fluid (Sánchez *et al.* 2010).

The involvement of ROS in chemotaxis is well known in other cell types such as neutrophils (Niethammer *et al.* 2009, Kuiper *et al.* 2011, Nathan & Cunningham-Bussell 2013). Since sperm chemotaxis is dependent on capacitation and ROS participate in the capacitation signaling via AMPc-PKA (O'Flaherty *et al.* 2004, 2006a, O'Flaherty 2015), which is the same pathway activated in the chemotaxis response (Teves *et al.* 2009) and

Figure 7 Sperm kinematic parameters are not affected by inhibitors/chelators of components of the chemotaxis signaling. (A) TAT-cAMP sponge, a chelator of cAMP; (B) H89, an inhibitor of PKA; (C) PF431396, an inhibitor of protein tyrosine phosphorylation, (D) BAPTA-AM, a chelator of the intracellular calcium; (E) DPI, an inhibitor of NOX. VCL, curvilinear velocity; VAP, mean trajectory velocity; VSL, straight line velocity. Results are presented as the mean $\pm$ S.E.M. of at least four experiments with ejaculates from three different horses.

that the sperm chemotaxis can be stimulated by nitric oxide (one of the nitrogen oxidative species) (Miraglia *et al.* 2007), it is probable that ROS also modulate the molecular mechanism of chemotaxis.

We investigated the participation of H_2O_2 in chemotaxis by two different strategies: (1) inhibition of chemotaxis by antioxidants; (2) activation of the chemotactic signal by the gradual intracellular increase of H_2O_2 , in sperm exposed to a gradient of H_2O_2 in the absence of P. Here we observed for the first time that, not only ROS are part of the AMPc-PKA signaling pathway that modulates sperm chemotaxis in stallion, but also that, similar to capacitation (O'Flaherty *et al.* 2004), the intracellular increase of ROS would be an event prior to the activation of this pathway.

The use of a concentration gradient of H_2O_2 combined with the addition of a cAMP chelator, led to the conclusion that H_2O_2 production occurs before the activation of the adenylate cyclase. Therefore, there should be some enzyme that produces ROS immediately after P binding to its receptor. In spermatozoa, NOX5 is an enzyme located in the plasma membrane of equine (Sabeur & Ball 2007) and human spermatozoa (Musset *et al.* 2012) that generates ROS by reducing NAD (NADPH). Moreover, this enzyme was shown to participate in the chemotaxis signaling of neutrophils (Woo *et al.* 2003) and several ROS-mediated signaling in spermatozoa (Aitken *et al.* 1992, Aitken *et al.* 1995, 1997, Ball *et al.* 2001, Armstrong *et al.* 2002, Sabeur & Ball 2006). In addition, NOX5 also has the ability to bind to calcium, which leads to the activation of oxidized

NADPH, and thus to the production of ROS (Tirone *et al.* 2010). Given that a chemotactic gradient of P stimulates an initial increase in intracellular calcium (Teves *et al.* 2009), and that NOX5 is activated immediately upon an increase in intracellular Ca^{2+} (Bánfi *et al.* 2004), this enzyme could mediate the production of intracellular ROS in chemotactic spermatozoa.

The results of this work led to propose a model of part of the molecular mechanism of sperm chemotaxis mediated by P. Thus, after P binding to its receptor, probably located in the plasma membrane, there is a first Ca^{2+} entry that activates NOX5, an integral membrane protein, generating an increase of ROS. This event, in conjunction with others, stimulates the increase of cAMP, followed by the activation of PKA and the subsequent PY, which after a series of intermediate steps involving Ca^{2+} , lead to the orientation of the flagellar movement toward the P source (Teves *et al.* 2009).

Declaration of interest

L C G is one of the inventors of the SSA device, and the Consejo de Investigaciones Científicas y Técnicas and the Universidad Nacional de Córdoba are the owners of the patent. The other authors have nothing to disclose.

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Author contribution statement

A M-I designed the study and experiments, performed experiments, analyzed data and wrote the article; E M D assisted A M-I in most of the experiments performed; C I M-B performed the WB for sperm PY; L L obtained and cryopreserved the equine semen samples; O L generated the TAT-cAMP sponge; R L gave us the inhibitor for flavoproteins, participated in the interpretation of data from these experiments, and provided fruitful comments to the manuscript. A M-V and C L T provided the inhibitors PF431396 for two tyrosine protein kinase and H89 dihydrochloride for PKA and gave advise about the design of these experiments and their interpretation. C N T, C L T, M G B helped us with results presentation and interpretation, providing fruitful comments to the manuscript; L C G designed the study and experiments, got financial support, wrote the article.

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