Effect of cariporide on ram sperm pH regulation and motility: possible role of NHE1

Stefania Muzzachi¹, Lorenzo Guerra¹, Nicola Antonio Martino^{1,2}, Maria Favia¹, Giuseppe Punzi¹, Fabio Silvestre³, Antonio Ciro Guaricci³, Maria Teresa Roscino³, Ciro Leonardo Pierri¹, Maria Elena Dell'Aquila¹, Valeria Casavola¹, Giovanni Michele Lacalandra³ and Elena Ciani¹

¹Department of Biosciences, Biotechnologies and Biopharmaceutics, University of Bari 'Aldo Moro', Bari, Italy,

²Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata, Foggia, Italy and ³Section of Veterinary Clinics and Animal Productions, Department of Emergency and Organ Transplantation, University of Bari 'Aldo Moro', Valenzano, Bari, Italy

Correspondence should be addressed to L Guerra; Email: lorenzo.guerra1@uniba.it

Abstract

Sperm motility, a feature essential for *in vivo* fertilization, is influenced by intracellular pH (pH_i) homeostasis. Several mechanisms are involved in pH_i regulation, among which sodium–hydrogen exchangers (NHEs), a family of integral transmembrane proteins that catalyze the exchange of Na⁺ for H⁺ across lipid bilayers. A preliminary characterization of NHE activity and kinetic parameters, followed by analysis of the expression and localization of the protein in ram spermatozoa was performed. NHE activity showed an apparent K_m for external Na⁺ of 17.61 mM. Immunoblotting revealed a molecular mass of 85 kDa. Immunolocalization pattern showed some species-specific aspects, such as positive labeling at the equatorial region of the sperm head. Cariporide, a selective NHE1 inhibitor, significantly reduced pH_i recovery (85%). Similarly, exposure to cariporide significantly inhibited different motility parameters, including those related to sperm capacitation. *In vitro* fertilization (IVF) was not affected by cariporide, possibly due to the non-dramatic, although significant, drop in motility and velocity parameters or due to prolonged exposure during IVF, which may have caused progressive loss of its inhibitory effect. In conclusion, this is the first study documenting, in a large animal model (sheep) of well-known translational relevance, a direct functional role of NHE on sperm pH_i and motility. The postulated specificity of cariporide toward isoform 1 of the Na⁺/H⁺ exchanger seems to suggest that NHE1 may contribute to the observed effects on sperm cell functionality.

Reproduction (2018) **155** 433–445

Introduction

At the end of its differentiation program, the sperm cell must acquire motility in order to be able to accomplish its mission, i.e. oocyte fertilization. Sperm motility, defined as a propagation of transverse waves along the flagellum in a proximal–distal direction, producing an impulse that pushes the spermatozoon in a straightforward manner through the male and the female reproductive system (Paoli *et al.* 2011), is a fundamental characteristic of sperm function and provides a reliable diagnostic and prognostic measure for male fertility. Sperm motility is achieved when the sperm cell has a fully developed and functionally active flagellum, or axoneme, and when the cell is capable of producing energy to fuel the movements. It is acquired in the male reproductive system, during the journey through the epididymis, where is called 'primary or activated motility' characterized by symmetrical tail movements

with low amplitude. Subsequently, it pursues through the female reproductive system, from the vagina and through the uterus to the fallopian tube, where it changes in 'hyperactivated motility' characterized by high amplitude and asymmetric flagellar bends occurring, in particular, when sperm cells reach the cumulus–oocyte complex (COC) in the ampullary tract (reviewed by Freitas *et al.* 2017).

As sperm is virtually devoid of transcription and translation due to highly condensed DNA and lack of endoplasmic reticulum (Baldi *et al.* 2002), activation or inhibition of specific signaling pathways play the most important roles in motility regulation. Indeed, the interaction between sperm and the environment created by the epididymis and the female reproductive tract are essential to trigger sperm motility. In this regard, the various processes that take place, at the sperm cell level, along the male and the female reproductive tract of mammals, are affected by pH levels in the several

luminal fluids (Martins *et al.* 2014, Sakkas *et al.* 2015, Ng *et al.* 2017) and disturbances of acid–base homeostasis have been associated with infertility or subfertility (reviewed by Martins *et al.* 2014). Thus, an interactive link exists between the ability of the sperm cell to maintain its correct intracellular pH and sperm motility, but this field of investigation is not yet extensively explored. It is known from the literature that two main categories of transmembrane proteins are involved in cell pH regulation, bicarbonate and proton carriers, respectively (Martins *et al.* 2014). The latter include a family of proteins, commonly termed sodium–hydrogen exchangers (NHEs), involved in the electroneutral exchange of intracellular H^+ with extracellular Na^+ according to the concentration gradient across the membrane (Freitas *et al.* 2017, Pereira *et al.* 2017). These proteins are widely expressed in several cell systems and are highly effective in pH_i regulation. In sperm cells, early studies on activity and role of sodium–hydrogen exchangers had been carried out in the sea urchin (Lee & Garbers 1986). More recently, NHEs have been shown to induce a raise of the intracellular pH, a mechanism which in turn activates CatSper, a cation channel of sperm cells that enables the entry of calcium, thus influencing glycolysis and the axoneme activity (Freitas *et al.* 2017, Pereira *et al.* 2017). Four isoforms (isoenzymes) of NHE have been identified so far on the sperm cell plasma membrane, namely NHE1, NHE5, mtsNHE (testis-specific NHE) and NHE10. The latter is also known as the sperm-specific NHE isoform (sNHE) as it is exclusively expressed in spermatozoa (Martins *et al.* 2014, Zhang *et al.* 2017) and has been already shown, similarly to mtsNHE, to play a role in affecting sperm motility (Wang *et al.* 2003, 2007, Liu *et al.* 2010, Zhang *et al.* 2017). Decreased motility, responsible for selective male infertility, and associated to sperm morpho-functional abnormalities, was also described in mice null for the organellar NHE8 isoform (Oberheide *et al.* 2017). No direct evidence for the involvement of the plasma membrane NHE1 and NHE5 isoforms in sperm motility has been provided so far. Between these two isoforms, NHE1 represents a better selection candidate for playing a role in the regulation of sperm function, as it is recognized as one of the most important systems involved in pH regulation in a wide variety of cell systems and is upregulated in different types of pathological conditions (Donowitz *et al.* 2013, Padan & Landau 2016). In addition, targeted disruption of the NHE1 locus in mouse was found to be associated with significant lifespan reduction in the offspring and variable effects on fertility according to the used breeding schemes either in homozygosity or heterozygosity (Bell *et al.* 1999). Furthermore, an indirect involvement of NHE1 in rat sperm motility was previously suggested by Woo *et al.* (2002)

through the use of ouabain, an inhibitor of the Na^+/K^+ -ATPase, that impairs the driving force for sodium. However, the adopted approach does not provide a definitive proof for the specific involvement of NHE1, as all Na^+/H^+ exchanger isoforms depend on the Na^+ gradient. To our knowledge, no studies have been reported to date of a direct involvement of NHE1 in sperm motility using its specific inhibitors.

In light of these considerations, and given the recent increased attention to NHE1-specific inhibitors, due to their use in the regulation of different cell functions with therapeutic purposes (Mihaila 2015), the aim of this study was to perform, for the first time, an evaluation of the effects of cariporide (HOE642), a documented selective inhibitor of NHE1, on sperm intracellular pH, motility and fertilizing ability. The study was performed on the ovine species as previous evidences supported it as an optimal animal model for translational studies in human reproductive physiology (Sutovsky *et al.* 1996).

Materials and methods

Ethics statement

Experiments were carried out at University of Bari, Southern Italy (42° north parallel) during reproductive seasons (September to November) 2014–2016. For animal handling and semen collection, the Principles for the Care and Use of Research Animals, promulgated by the European Community, were followed. Semen collection was carried out with the rams owners' consent, as part of the routine activities at the Section of Veterinary Clinics and Animal Production, Department of Emergency and Organ Transplantation, which is a recognized center for livestock semen collection and storage, authorized by the Apulian Regional Authority (n. 311/ICA 29/11/2004). As such, no specific approval for this study was required at the Animal Research Ethical Committee ('Comitato Etico per la sperimentazione animale', CESA) of University of Bari.

Semen collection

Fresh ejaculates were collected from three rams with proven general and reproductive health by using an artificial vagina. The rams were kept under standard housing and feeding conditions at the Teaching Veterinary Hospital of the University of Bari, Italy. After measuring volume, density and coarse motility under light microscopy (80–90%), semen was diluted 1:10 with a non-capacitating medium in order to avoid interference with bicarbonate-dependent pH regulatory mechanisms. A modified bicarbonate-free Sperm Analysis Medium (Cox *et al.* 2006), namely NaCl medium (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Glucose, 10 mM HEPES, pH 7.4 with NaOH), was used. Motile spermatozoa were separated from non-motile ones and seminal plasma by the swim-up technique. Briefly, 100 μ L of semen were incubated with 1 mL of NaCl medium at 37°C for 1 h in a humidified incubator. After incubation, motile

spermatozoa that were present into the supernatant were collected and used for subsequent analyses.

Western blot

Spermatozoa (21×10^6) were centrifuged at $16,000g$ for 5 min, homogenized in lysis buffer (110 mM NaCl, 50 mM TRIS, 0.5% (v/v) Triton X-100, 0.5% (v/v) Igepal CA-630, pH 8 added with 0.2% (v/v) protease inhibitor mixture Sigma-Aldrich P8340), sonicated for 10 s and placed overnight at -80°C . Then, samples were heated at 37°C for 30 min and centrifuged at $16,000g$ for 10 min. Supernatant protein concentration was measured by the method of Bradford (1976). Ghosts preparations were obtained from fresh ram blood. Approximately, 5 mL of ram blood was diluted in 20 mL of medium (120 mM NaCl, 10 mM EDTA, 5 mM sodium citrate and 5 mM Tris-HCl, pH 7.4) and centrifuged at $1500g$ for 10 min. The pellet was washed twice by gentle resuspension in 20 mL of PBS, pH 7.4, and subsequent centrifugation at $1500g$ for 15 min, and then it was resuspended and incubated in 5 mM phosphate buffer. After 1 h of incubation, the sample was centrifuged at $13,000g$ for 30 min at 4°C and the pellet was resuspended in 5 mM phosphate buffer and further centrifuged at $13,000g$ for 30 min at 4°C . The final pellet was resuspended in distilled water. Protein concentration was measured as mentioned earlier. For both sperm proteins and ghosts, a $30\mu\text{g}$ aliquot was diluted in Laemmli buffer, heated at 100°C for 5 min and separated by 9% (v/v) Tris/HCl SDS/PAGE gel electrophoresis under reducing conditions. The separated proteins were transferred to Immobilon P (Millipore) for immunoblotting (Favia et al. 2014). The used antibodies were a monoclonal anti-NHE1 antibody (1:400 dilution), which specifically binds the amino-terminal domain (ab58304; Abcam) and an anti-NHE1 polyclonal antibody (1:400 dilution) against the C-terminus epitope (AB3081; Chemicon by Merck Millipore). After washing, blots were incubated with anti-mouse or anti-rabbit secondary antibodies conjugated to peroxidase (1:2000 dilution). Immunocomplexes were detected with the ECL plus reagent (GE Healthcare Life Sciences).

Immunofluorescence

Suspensions of 5×10^5 spermatozoa were seeded on round glass coverslips treated with polylysine. Then, sperm cells were fixed in 4% (v/v) paraformaldehyde for 20 min and permeabilized using 0.1% (v/v) Triton X-100 in PBS for 10 min, blocked using 0.1% (w/v) gelatin in PBS for 10 min. Afterward, spermatozoa were incubated for 2 h with primary antibodies, the monoclonal anti-NHE1 (1:200 dilution) or the polyclonal anti-NHE1 (1:200 dilution) previously adopted in the Western blot analysis. Spermatozoa were subsequently incubated with goat anti-mouse or anti-rabbit IgG secondary antibodies conjugated to Alexa Fluor 488 (1:1000 dilution) for 1 h. Coverslips were then mounted onto slides using ProLong Gold Antifade Reagent with DAPI (Invitrogen) and examined with Leica DMRXA microscope. Specimens were viewed through a $100\times$ oil immersion

objective and images were acquired by NIKON digital camera DXM1200.

Intracellular pH spectrofluorimetric analysis

Spermatozoa were incubated with $5\mu\text{M}$ BCECF-AM (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester) for 30 min at room temperature under constant agitation in NaCl medium and then centrifuged at $500g$ for 5 min. The pellet was resuspended in NaCl medium, added with 40 mM NH_4Cl and incubated for 10 min. In the medium, NH_4Cl dissociates to NH_3 , which readily crosses the cell membrane and, due to binding to H^+ , leads to a rapid intracellular alkalization. The suspension was then centrifuged at $500g$ for 5 min, and the pellet was incubated for 15 min in a Na^+ -free medium (150 mM TMACl (tetramethylammonium chloride), 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM Glucose, 10 mM HEPES, pH 7.4 adjusted with KOH), thus removing external NH_4Cl and leading to intracellular acidification via the reverse NH_3 extrusion process. After incubation, sperm cells were centrifuged at $500g$ for 5 min and the pellet was resuspended in Na^+ -free medium. To evaluate NHE activity, spermatozoa (10×10^6) were added in a quartz cuvette containing the NaCl medium at different Na^+ concentrations (15, 30, 75, 150 mM) and placed into the spectrofluorometer (Cary Eclipse Varian). The fluorescence was measured at excitation wavelengths of 450 and 500 nm and emission wavelength of 535 nm. Intracellular pH was estimated from the ratio of BCECF fluorescence calibrated by using the K^+ nigericin method (Thomas et al. 1979, Montrose et al. 1987). Briefly, for the calibration curve, sperm cells were incubated with $5\mu\text{M}$ BCECF-AM and $5\mu\text{M}$ nigericin in a KCl medium (110 mM KCl, 20 mM NaCl, 1 mM CaCl_2 , 1 mM MgSO_4 , 1 mM KH_2PO_4 , 18 mM D-Glucose, 20 mM HEPES, pH 7.4) for 30 min. After incubation, sperm cells were centrifuged at $500g$ for 5 min and the pellet was resuspended in the KCl medium added with nigericin; the fluorescence was then measured in the KCl medium at different pH values (6, 6.5, 7 and 7.5). To estimate the values for the kinetic parameters V_{max} and K_m , we adopted the Woolf-Augustinsson-Hofstee equation, a linearized form of the Michaelis-Menten equation that can be advantageously used to plot $(V/[S])$ against V , where V represents ($\Delta\text{pH}/\text{min}$) and S represents the substrate (in our case, the extracellular Na^+ concentration). In the obtained plot, the y intercept represents V_{max} , the x intercept represents V_{max}/K_m and the slope represents $-K_m$. Data were expressed as mean $\pm$ standard error (s.e.). Statistical comparisons were made using the unpaired Student's *t*-test, adopting a significance threshold of $P < 0.05$.

NHE1 inhibitors

An extensive literature review on specific NHE1 inhibition studies provided evidence on the common use of dimethylamiloride (DMA) and cariporide (HOE642), respectively, an amiloride-derivative and a benzoylguanidine derivative. For both inhibitors, concentration ranges between nanomolar to micromolar are described. As we worked on an animal species for which, to the best of our knowledge,

no previous studies on NHE inhibition had been published in any cell system, for both inhibitors, we adopted micromolar concentration values well above the reported DMA and cariporide IC₅₀ for NHE1 and below the cariporide IC₅₀ for NHE5 (Masereel *et al.* 2003).

Protein sequence alignment, 3D modeling and docking analyses

NHE1, NHE5, NHE8 and NHE10 orthologous sequences were sampled from the *refseq* database by using *blastp* and the human NHE1, NHE5, NHE8 and NHE10 sequences as query sequences for each subgroup, respectively. The sampled sequences were aligned by using *ClustalW* implemented in the *Jalview* suite.

I-tasser and *pGenTHREADER* were used for identifying the sequences of the proper crystallized template structures and for building a pairwise sequence structure alignment for the following 3D modeling step according to protocols described in Pierri *et al.* (2010). *SwissPDBViewer* was used for generating 3D models of NHE1 and NHE10. *PyMOL* was used for comparative analyses of the generated 3D models and the crystallized structure used as template for protein modeling according to protocols described in Pierri *et al.* (2010, 2016).

Autodock was used for docking cariporide in NHE1- and NHE10-binding regions according to protocols described in Pierri *et al.* (2010) and Todisco *et al.* (2016). The docking analyses were performed using a gridbox, whose center was in correspondence of the TI⁺ ion. The employed gridbox consists of 35×35×35 gridpoints in the x–y–z Cartesian space. The distance between each gridpoint of the gridbox was set to 0.281 Å. The parameter '*rmstol*' was set to 1.5; the parameter '*ga_pop_size*' was set to 50, the parameter '*ga_num_evals*' was set to 2.5×10^5 , the parameter '*ga_num_generations*' was set to 2.7×10^4 . The number of top individuals to survive to the next generation was set to 5 and a ranked cluster analysis of the 50 cariporide poses generated was performed.

Sperm motility assessment by Computer-Assisted Sperm Analysis (CASA)

The effect of cariporide on sperm motility was analyzed by the HTM-IVOS Computer-Assisted Sperm Analyzer (CASA), version 12.3 (Hamilton-Thorne Biosciences, MA, USA). Semen samples (0.2 mL/straw) from a single ejaculate frozen at the concentration of 1×10^8 sperm cells/mL were rapidly thawed in water bath at 38.5°C for 30 s. Subsequently, cells underwent the swim-up procedure in NaCl medium. After *in vitro* exposure under inhibiting or control conditions, the supernatants (0.6 mL) were recovered and centrifuged at 300 g for 10 min. The pellets were resuspended in 100 µL NaCl medium. CASA analyses were conducted at 38.5°C by loading 4 µL of sperm samples on specific slide chambers, 20 µm in depth (Leja 4, Leja Products B.V., The Netherlands). For each sample, 3 independent cameras were used and a total of 24 random fields (8 fields/camera) were counted. The following parameters were measured: the percentage of motile spermatozoa (total motility, %); the percentage of progressively motile spermatozoa (progressive motility, %); the

average path velocity (VAP, µm/s), calculated as the curvilinear trajectory of the sperm head; the straight-line rectilinear velocity (VSL, µm/s), calculated as the velocity of the sperm head along the trajectory between the first and the last spotted position; the curvilinear velocity (VCL, µm/s), calculated as the velocity of the sperm head along the total covered distance and the amplitude of the lateral head displacement (ALH, µm). Furthermore, sperm velocity distribution was analyzed by identifying four sperm cell movement subcategories based on ram-specific VAP cutoff, as reported in the HTM-IVOS CASA system, version 12.3 software: percentage of rapid cells (fraction of cells moving with VAP >75 µm/s; rapid, %); percentage of medium speed cells (fraction of cells moving with VAP values ranging from 21.9 to 75 µm/s; medium, %); percentage of slow cells (fraction of cells with VAP <21.9–75 µm/s; slow, %) and percentage of static cells (fraction of cells not moving at all; static, %). Sperm motility parameters were defined as previously reported in the IVOS software manual, and in Mortimer and Maxwell (1999), Palacin *et al.* (2013), Najafi *et al.* (2017). Statistical comparisons were made by using the unpaired Student's *t*-test.

IVF assay

Ovaries from juvenile (<6 months of age) sheep were recovered at local slaughterhouses in accordance with the Council Directive 89/556/ECC and subsequent modifications. Ovaries were processed by the slicing procedure and only cumulus–oocyte complexes (COCs) with intact cumulus cell layers and a homogeneous cytoplasm were selected. *In vitro* maturation (IVM) was performed as previously reported (Martino *et al.* 2016). Medium TCM-199 with Earle's salts, buffered with 4.43 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 33.9 mM sodium bicarbonate and supplemented with 0.1 g/L L-glutamine, 2 mM sodium pyruvate, 2.92 mM calcium-L-lactate pentahydrate (Fluka 21175; Serva Feinbiochem), 50 µg/mL gentamicin, 10% (v/v) fetal calf serum (FCS), 10 µg/mL ovine follicle-stimulating hormone (FSH) and 20 µg/mL ovine luteinizing hormone (LH), 1 µg/mL 17 beta estradiol was used. COCs were placed in 400 µL of medium/well of a four-well dish (Nunc Intermed, Roskilde, Denmark) covered with pre-equilibrated lightweight paraffin oil and cultured for 24 h at 38.5°C under 5% CO₂ in air. After IVM culture, oocytes underwent partial cumulus cell removal by incubation in TCM 199 with 20% FCS and aspiration in and out of finely drawn glass pipettes.

Fresh sperm, collected as described earlier, or frozen semen samples (0.2 mL/straw) from a single ejaculate at the concentration of 1×10^8 sperm cells/mL, underwent the swim-up procedure (Martino *et al.* 2016) in NaCl medium under inhibiting or control conditions. The final sperm concentration of 1×10^6 sperm cells/mL was added to 400 µL of IVF culture medium (HEPES-buffered Tyrode's-lactate pyruvate medium containing 10 µg/mL heparin) containing *in vitro* matured oocytes (see above). Spermatozoa and oocytes (5–20 oocytes per well) were kept together for 24 h at 38.5°C under 5% CO₂ in air. On the day after IVF, oocytes were assessed for signs of fertilization. To evaluate nuclear chromatin, oocytes underwent cumulus and corona cell removal and were fixed

in 3.8% buffered formaldehyde solution, stained with 2.5 $\mu\text{g}/\text{mL}$ Hoechst 33258 (Sigma) in 3:1 (v/v) glycerol/PBS and mounted on microscope slides. Oocytes were observed under an epifluorescence microscope (Nikon Eclipse 600; 400 $\times$ magnification) equipped with B-2 A (346 nm excitation/460 nm emission) filter. Normal fertilization was defined by the presence of two pronuclei (PN) and two polar bodies (PB). Oocytes found cleaved (two- to four-cell stage), showing two to four regular-shaped blastomeres with a normal nucleus within each blastomere, were also considered as normally fertilized. Presence of a swollen sperm head, a single PN with signs of the sperm cell in the cytoplasm, and abnormal cleavage (irregular shape blastomeres with irregular number of nuclei) were considered to represent retarded, arrested or abnormal fertilization and were grouped together as abnormal. Parthenogenetically activated oocytes showing one PN and one PB and oocytes showing the metaphase II with or without the intact sperm cell were classified as unfertilized. Nuclear chromatin status of other unfertilized oocytes was classified as follows in relation to the stage at which meiotic development was interrupted: germinal vesicle (GV), metaphase to telophase I (MI) and metaphase II (MII) with or without the chromatin of the first PB. Oocytes showing either multipolar meiotic spindle or irregular chromatin clumps or no chromatin were considered as abnormal. Oocytes with fragmented or shrunken cytoplasm were classified as degenerated. Oocyte fertilization rates were compared between treated and control groups by chi-square test with the Yates correction for continuity.

Results

Functional characterization of NHE activity in ram spermatozoa

Before proceeding with specific inhibition tests, the overall NHE activity in the sperm cell was analyzed, as literature data are lacking in this species. NHE activity was evaluated by pH_i measurement carried out using the BCECF-AM fluorescent dye. The calibration curve of the fluorescence ratio as a function of pH_i is shown as Fig. 1A. As can be observed, the curve is linear over a pH_i range of 6.0–7.5, similarly to what was previously reported in other cell systems (Wang *et al.* 2003). The calibration curve was used in all subsequent experiments to convert fluorescence ratios (500/450 nm) into pH_i values. Consistently with data reported in sperm cells of other mammalian species (Mannowetz *et al.* 2012), the resting pH_i in fresh ram spermatozoa under non-capacitating conditions in a medium containing NaCl at physiological concentration (150 mM) was 7.04 ± 0.03 , as evaluated in 22 independent experiments. At this pH value, the Na^+/H^+ exchanger is normally quiescent. As it rapidly becomes activated as soon as the acidity of the cytoplasm increases, we therefore characterized the protein activity, namely its ability to extrude H^+ ions, in non-capacitated sperm cells subjected to intracellular acidification by an ammonium chloride pulse (see 'Materials and methods' section). After NH_4Cl acid

Figure 1 (A) Calibration curve of intracellular pH_i . Ram spermatozoa were loaded with the fluorescence dye BCECF-AM, and the high- K^+ /nigericin technique was used to clamp pH_i to extracellular pH (pH_e). pH_e was varied from 6.0 to 7.5. Data from 8 experiments performed on 3 rams were linearly fitted. (B, C and D) NHE activity in ram spermatozoa. Panel B presents a typical trace of pH_i recovery when spermatozoa were resuspended in Na^+ medium (150 mM) after an acid load. The effect of change in external Na^+ on pH_i recovery is shown in panel C. A decrease of $[\text{Na}^+]_e$ from 150 to 15 mM slowed down NHE1 rate. Panels B and C represent the results obtained from several experiments carried out on sperm from three different rams. A Woolf–Augustinsson–Hofstee plot of the same data is shown in panel D.

load (40 mM, for 10 min), internal pH was 6.72 ± 0.008 ($n=108$ independent experiments). In all subsequent analysis, the Na^+/H^+ exchange activity was therefore evaluated when reaching the above pH value. Recovery from acid load was observed when spermatozoa were suspended in the 150 mM NaCl medium (Fig. 1B), with a rate of Na^+/H^+ exchange of 0.042 $\Delta\text{pH}/\text{min}$. On the contrary, no recovery of pH_i was observed when spermatozoa were suspended in a Na^+ -free medium, thus demonstrating that no proton extrusion pathway, with significant transport rate, exists in the absence of sodium (data not shown).

NHE kinetic parameters in sperm cells

In order to provide indirect evidence that NHE plays a role in intracellular pH regulation in the sperm cell, we performed the same experiments as described earlier in the presence of increasing extracellular Na^+ concentrations. In detail, the initial pH_i recovery rate was measured in the presence of 15 ($n=20$ independent experiments), 30 ($n=8$ independent experiments), 75 ($n=6$ independent experiments) and 150 ($n=25$ independent experiments) mM extracellular sodium (Fig. 1C). Significant decreases of NHE activity were observed at the lower extracellular

Na^+ concentrations (15 and 30 mM) compared to the physiological concentration. Analysis of the same data by the Woolf–Augustinsson–Hofstee equation showed an apparent K_m value for external Na^+ of 17.61 mM in the sperm cell (Fig. 1D).

Protein expression and localization in ram spermatozoa

As we have decided to conduct the study of the functional mechanism of NHE1 on the ovine sperm model for its reproductive physiology features closer to those of human sperm compared with laboratory animals, it was necessary to preliminarily analyze NHE1 expression on ovine sperm. In order to increase the specificity of the analysis, and due to the lack of NHE1 antibodies specifically raised against cell antigens of the ovine species, the study was conducted in a double confirmation mode, as two anti-NHE1 antibodies, for two different regions of the same protein, were used, namely the polyclonal anti-NHE1 against the intracellular C-terminus adopted in the previous study by Woo *et al.* (2002), and a monoclonal antibody raised against the extracellular N-terminus (Yang *et al.* 2013). Furthermore, *in silico* analysis of NHE1, NHE5, NHE8 and NHE10 protein sequences highlighted remarkable variability in length and amino acid composition at both the N-terminus and at the C-terminus (Supplementary Figs 1 and 2, respectively, see section on supplementary data given at the end of this article) among the four isoforms, that is consistent with the proposed high selectivity of the two antibodies adopted in this study vs NHE1. Indeed, both antibodies detected a single band with an apparent molecular mass of 85 kDa (Fig. 2A). A band of similar size, but weaker intensity, was detected in ram ghosts (red blood cells membranes), which were used as positive controls, due to the previously reported notion that they express NHE1 as a major sodium–proton exchanger isoform in several mammalian and not mammalian species (Pedersen & Cala 2004).

The immunofluorescence analysis revealed, consistently for both antibodies, the presence of positive signals at the equatorial region of the sperm head, in the mid-piece and the proximal segment of the flagellum (Fig. 2B). The lack of immunoreactivity in sperm cells, where the primary antibody was omitted, confirmed the labeling specificity (Fig. 2C).

Effects of Na^+/H^+ exchanger inhibitors on sperm intracellular pH regulation

To add evidence for the possible involvement of the NHE isoform 1 in sperm pH_i regulation, we adopted two selective NHE1 inhibitors, DMA and cariporide. Data were obtained in sperm cells exposed to acid load, and then incubated for 30 min in Na^+ -free medium in

Figure 2 NHE1 expression and localization on ram sperm cells. (A) Representative Western blot of ram whole sperms and red blood lysates (ghosts) using a monoclonal anti-NHE1 antibody (1:400 dilution), which specifically binds the amino-terminal domain and a polyclonal anti-NHE1 antibody (1:400 dilution) against the C-terminus epitope. The two antibodies detected an apparent band of approximately 85 kDa in both cell types. (B) Representative photomicrographs of NHE1 localization in ram sperm cells investigated through immunofluorescence by using the same anti-NHE1 polyclonal antibody (1:200 dilution) against the C-terminus domain (left panel) and the monoclonal antibody anti-NHE1 (1:200 dilution) against the N-terminus domain (right panel) as used in the Western blot analysis. Intense staining was observed on the mid-piece surface of the sperm flagellum, together with a weaker signal at the equatorial region of the sperm head. Nuclei are counterstained with DAPI (blue). (C) As negative control, spermatozoa were incubated only with FITC-conjugated secondary antibodies. Only DAPI-stained nuclei were detectable. Scale bar: 10 μm .

the presence of each inhibitor, followed by incubation under physiological Na^+ concentrations. As shown in Fig. 3, both inhibitors significantly reduced pH_i recovery, with cariporide displaying a stronger effect (85% inhibition with $P < 0.001$ vs 50% inhibition with $P < 0.01$, respectively).

Figure 3 Effect of DMA and cariporide treatments on cytoplasmic pH recovery. The effect of DMA (10 μM) and cariporide (10 μM) on the rate of pH_i recovery after acidification of non-capacitated spermatozoa was evaluated by the ammonium pulse technique. Sperm cells were incubated with DMA or cariporide for 30 min in the Na⁺-free medium and then added in NaCl medium (150 mM). Results are means ± s.e. from several experiments carried out on sperm from three different rams.

Protein sequence alignment, 3D modeling and docking results

From multiple sequence alignment of sodium-proton exchanger plasma membrane isoforms known to be expressed in sperm cells, NHE1 and NHE5 orthologs appear to share 85–90% of identical residues, whereas a greater variability is observed among NHE10 orthologs, sharing the 66–70% of identical residues. NHE1 and

NHE5 paralogs share the 40–50% of identical residues, whereas NHE1/5 shares only the 20–25% of identical residues with NHE10. Notably, NHE10 sequences have a C-terminus longer than the C-terminus observed in NHE1 and NHE5 paralogs (Supplementary Fig. 2). In general, NHE1, NHE5 and NHE10 share a common overall structure allowing them to work as Na⁺/H⁺ antiporters. In particular, NHE1/5/10 shows a well-conserved substrate ion-binding site and polar channel with the *Pyrococcus abyssi* Na⁺/H⁺ antiporter (PaNhaP), whose full-length sequence shows the 23–27% of identical residues with NHE1, NHE5 and NHE10. From the PaNhaP crystal structure, it was observed that residues coordinating directly the ion substrate were E73 (helix 3) and D159 (helix 6), whereas D130 (helix 5) appeared to interact with the ion substrate via a water molecule. T129 (helix 5) and S155 (helix 6) also were proposed to participate in ion substrate coordination. A second channel crucial for Na⁺/H⁺ antiport function was highlighted and consisted of residues D93, N158 and the ion pair E154/R337 that together with T129 separates the cavity hosting the ion substrate-binding site from the polar channel (Goswami *et al.* 2011, Wohler *et al.* 2014).

From multiple sequence alignment, it is observed that E73 from PhNhaP has an acidic counterpart in NHE1 (HsaNHE1, D172; OarNHE1, D172) and NHE5 (HsaNHE5, D118; OarNHE1, D120) paralogs, whereas in NHE10 that acidic residue is replaced by a hydrophilic residue (HsaNHE10, T90; OarNHE10, T109). It is also observed that D159 is conserved in NHE1 (HsaNHE1, D267; OarNHE1, D267) and NHE5 (HsaNHE5, D214; OarNHE5, D216), whereas in NHE10, it is replaced with

Figure 4 An extract of the multiple sequence alignment is reported by using the Jalview Zappo color style. Labels of residues involved in the ion substrate binding site and in the polar channel are reported upon the alignment. “*” indicates the two residues playing a crucial role in the affinity for cariporide.

a hydrophilic residue (HsaNHE10, S179; OarNHE10, S198) (Fig. 4).

The resulting 3D models generated by using *SwissPDBViewer* include the NHE1, NHE5 and NHE10 portions involved in the antiport mechanism and consisted of 13 helices. In each of the 3 antiporters, helices H1–H3, H7–H10 form the dimer interface, whereas helices H4–H6, H11–H13 form a polar channel according to Goswami *et al.* (2011) and Wohler *et al.* (2014). For a representation of the dimers, refer to Supplementary Fig. 3.

PyMOL was used for superimposing the generated 3D models with the crystallized Na⁺/H⁺ antiporter from *P. abyssi* (PhNhaP). Thus, it was possible to obtain TI⁺ ion and water molecules crucial for protein function in the proper binding site of the human paralogs through the superimposition with the PhNhaP. Thanks to structure superimposition it was observed that residues within 4 Å from TI⁺ ion and cariporide (aligned with those proposed as crucial for PaNhaP) in the human NHE1 are D172, V237, D238, V240, E262, S263, N266, D267 (see the corresponding conserved NHE5 residues from the alignment of Fig. 4), whereas in the human NHE10 the corresponding residues are T90, S149, D150, M152, E174, S175, T178, S179 (Fig. 5). Notably, other residues important for cariporide recognition (from docking analyses) in NHE1 are K384 and E391, whereas in NHE10, the corresponding residues are K283 and E288 (Fig. 5).

Figure 5 3D comparative models of the human NHE1 and NHE10. The top view of a single monomer of NHE1 (left panels) and NHE10 (right panels) are reported in cartoon representation. Polar channels formed by helices H1–H3 and H7–H10 are reported in gray (NHE1) and cyan (NHE10), whereas helices H4–H6 and H11–H13 at the dimer interface are reported in blue (NHE1) and magenta (NHE10) (Supplementary Fig. 3). Residues within 4 Å from cariporide (yellow sticks) and TI⁺ ion (brown sphere) are reported in sticks (white and blue for NHE1; cyan and magenta for NHE10).

Effect of cariporide on sperm motility

As cariporide displayed a stronger inhibiting effect compared with DMA, and considering that the aforementioned protein alignment data supported the knowledge of its selectivity toward NHE1, this inhibitor was selected for being used in subsequent motility and fertility experiments.

The effect of cariporide on sperm cell motility parameters was assessed by CASA analysis at the concentration of 20 µM and at different exposure times (60, 120 and 180 min). Exposure to cariporide induced a statistically significant reduction of total motility at 120 and 180 min ($P < 0.05$; Fig. 6A), whereas progressive motility was not affected at any examined time (Fig. 6B). However, by examining sperm samples into four subcategories of progressive sperm movement (rapid, medium, slow and static), it was possible to observe that the cariporide treatment significantly reduced the percentage of rapid cells ($P < 0.05$; Fig. 6C) and increased the percentage of medium ($P < 0.01$), slow (from $P < 0.05$) and static cells ($P < 0.05$; Fig. 6D, E and F). Similarly, sperm velocity sub-parameters (VAP, VSL, VCL) were significantly reduced after 60 min ($P < 0.0001$; Fig. 7A, B and C). At 180 min exposure, only VCL was reduced ($P < 0.01$; Fig. 7C). Furthermore, cariporide induced a reduction of ALH after 120- ($P < 0.05$) and 180 ($P < 0.01$)-min exposure (Fig. 7D). Notably, VSL, VCL and ALH are also considered as capacitation-related sperm parameters.

Effects of cariporide on sperm fertilizing ability

Assessing the effects of cariporide on IVF rate, under the same conditions adopted for the characterization of the exchanger activity and sperm motility testing, was a hard technical issue as the fertilization protocol requires specific media containing bicarbonate. Fertilization experiments were initially carried out using fresh semen. Poor fertilization rates with no significant differences between cariporide-exposed and control samples were observed (Table 1, left column). This result was thought to be related to poor capacitation rate occurring during sperm swim-up in NaCl medium. Therefore, experiments were repeated using frozen/thawed sperm, which is known to 'per se' display a fraction of capacitated cells (Perez *et al.* 1996). As expected, the fertilization rates with frozen/thawed sperm were significantly increased ($P < 0.05$) compared with fresh sperm. However, even in this case, no significant differences were found between cariporide-exposed and control samples (Table 1, right column).

Discussion

All our experiments were performed in the absence of bicarbonate, which allowed us to exclude the

Figure 6 Time-dependent effect of cariporide on total and progressive motility of ram sperm cell analyzed at three different exposure times (60, 120 and 180 min) by Computer-Assisted Sperm Analysis (CASA). The reduction of the sperm motility was assessed by analyzing total motility (A), progressive motility (B) and four subcategories of progressive movement: rapid, medium, slow and static (C, D, E and F).

involvement of bicarbonate-dependent alkalizing mechanisms known from the literature to be involved in cell pH regulation (Martins *et al.* 2014). As such, we were able to evaluate, for the first time in sperm cells, the kinetic parameters of NHE, and to highlight the importance of the sodium gradient on the export of H^+ . Moreover, the observed sodium affinity for the NHE-binding site was consistent with previous evidences of NHE exhibiting K_m values ranging 5–50 mM in other cell types and other species (Masereel *et al.* 2003, Goswami *et al.* 2011, Wohler *et al.* 2014).

Most of the literature on NHE1 expression in different cell systems from various mammalian species describes a band of approximately 95–110 kDa (Leung *et al.* 2001, Slepko *et al.* 2005, Cuello *et al.* 2007), the different molecular weights likely reflecting distinct glycosylation levels. However, consistent with our results, a ~85 kDa

NHE1 protein was observed in rat in palmar skin and renal inner medulla (Nejsum *et al.* 2005) and in immortalized proximal tubule cells (Carraro-Lacroix *et al.* 2006). Similarly, a NHE1 band of ~80 kDa was described in rat efferent ductal and cauda epididymal epithelial cells (Leung *et al.* 2001), rat pancreas (Roussa *et al.* 2001) and adult rat ventricular myocytes (Cuello *et al.* 2007). A lighter NHE1 band (70 kDa) was also observed in bovine, basolateral membrane of fresh corneal endothelial cells (Rimmer 1999). Notably, Woo *et al.* (2002) firstly reported the expression of NHE1 in rat sperm cells through Western blot analysis, highlighting the detection of a single 95 kDa band. On the other hand, Wang *et al.* (2003) reported, in mouse sperm cells, a single band of about 130 kDa for the NHE10 isoform. Based on the above-mentioned results, the protein observed in the present study may reflect low glycosylation levels

Figure 7 Effects of cariporide on CASA velocity sub-parameters (VAP, VCL, VSL) (panels A, B and C), and ALH, a capacitation-related parameter (D). The analysis was conducted at 38.5°C. For each sample, three independent cameras were used and a total of 24 random fields (8 fields/camera) were counted. Statistical comparisons were made using the unpaired Student's *t*-test, adopting a significance threshold of $P < 0.05$.

Table 1 Effects of cariporide on the fertilizing ability of ram sperm cells.

Cultured oocytes, <i>n</i>	23	24	50	70
Swim-up	CTRL	Cariporide	CTRL	Cariporide
Sperm type	Fresh	Fresh	Frozen	Frozen
Swim-up medium	NaCl	NaCl	NaCl	NaCl
IVF medium	TALP – FERT	TALP – FERT	TALP – FERT	TALP – FERT
Sperm (/mL)	1 × 10 ⁶	1 × 10 ⁶	1 × 10 ⁶	1 × 10 ⁶
Oocytes evaluated after IVM, <i>n</i>	23	24	45	70
Germinal vesicle	7	2	9	19
Metaphase I to Telophase I	5	7	2	6
Metaphase II	7	8	18	18
Abnormal	0	1	1	4
Activated (/eval post IVM: %)	2	1	1	4
Maturation rate (%)	11 (47.8)	14 (58.3)	32 (71.1)	37 (52.9)
2PN2PB (/matured: %)	1 (9.1)	3 (21.4)	8 (25)	12 (32.7)
2PN2PB (/eval post IVM: %)	1 (4.3)	3 (12.5)	8 (17.8)	12 (17.1)
1PN + spz dec (/fert: %)	0	1	0	0
>2PN (/fert: %)	1	1	6	7
Fertilization rate (/matured: %)	2 (18.2)	5 (35.7)	14 (43.7)	19 (51.3)
Fertilization rate (/eval post IVM: %)	2 (8.7) ^a	5 (20.8)	14 (31.1) ^b	19 (27.1)

Chi-square test: ^{a,b}*P* < 0.05.

IVF, *in vitro* fertilization; IVM, *in vitro* maturation; PB, polar body; PN, pronucleus; spz, spermatozoa; TALP, Tyrode's-lactate pyruvate medium.

and/or other post-transcriptional modifications in ram sperm cells NHE1, likely a species-specific feature, as no difference was observed between sperm and red blood cells in sheep.

Concerning the localization at the flagellum level, the pattern observed in this study was clearly different from that reported in mouse sperm for the two described sperm-specific (sNHE) and testis-specific (mtsNHE) isoforms, both observed only in the main piece (Wang *et al.* 2003, Liu *et al.* 2010) thus adding evidence to the notion of specificity of the antibodies adopted here. Similar to what was observed by Woo *et al.* (2002) in rat sperm cells, we observed the expression of NHE1 at the mid-piece and in the proximal segment of the flagellum, while the presence of NHE1 on the sperm head is described here for the first time. Further, the use of specific NHE1 inhibitors, DMA and cariporide, allowed us to evidence, for the first time, the functional expression of this NHE isoform in sperm cells through evaluation of its involvement in intracellular pH regulation. Moreover, the *in silico* evidence that the human NHE1 protein has a remarkably different protein sequence and organization compared to sNHE suggested that both cariporide and DMA may be inactive on that isoform, for which published inhibition studies are still missing. The observed difference in the NHE1 inhibition of intracellular pH regulation between the two drugs adopted in the present study likely reflects their chemical nature (Mihaila 2015). Indeed, cariporide has been previously demonstrated to be a more potent, specific and selective inhibitor of NHE1 than other NHE inhibitors of the amiloride class (Siyanov & Baltz 2013).

It is known from the literature that Na⁺/H⁺ antiporter proteins have in common a substrate ion-binding site (for sodium) and a polar channel deeply involved in the binding and Na⁺/H⁺ antiport (Wohlert *et al.* 2014).

Wohlert and coworkers crystallized the sodium-proton antiporter from PaNhaP as dimer and each monomer was complexed with a thallium (Tl⁺) ion, another substrate of PaNhaP, for gaining new insights about Na⁺ binding. The ion-binding site results are accessible from the cytoplasm but not from the extracellular side, which shows the inward-open conformation of PaNhaP (Wohlert *et al.* 2014).

For investigating the affinity of cariporide to NHE paralogs, we built multiple sequence alignments and 3D comparative models of human and ovine NHE1, NHE5 and sNHE (NHE10), the only NHE isoforms identified so far on the sperm plasma membrane (Martins *et al.* 2014). From multiple sequence alignments and comparative models, it was possible to observe that acidic residues (D172 and D267, human NHE1 numbering) conserved in PaNhaP, NHE1 and NHE5, involved in the binding of Tl⁺ and cariporide, are replaced with hydrophilic residues in NHE10 (T90 and S179). From docking analyses, it is possible to see that above all the replacement of D172 and D267 with T90 and S179, respectively, could be responsible for lowering the affinity of NHE10 for cariporide. In general, we can speculate that the arrangement of residues D172, D267, E391 and K384 in NHE1 (and NHE5) appears to establish stronger interactions with cariporide than those observable with T90, S179, E288 and K283 in NHE10. It is possible to observe several hydrophobic residues around D172, D267 and D238 in human NHE1 and their counterpart in human NHE5, namely D185, D214, but not D118 (Fig. 4). Most likely, the presence of residues I170 and A173 close to D172 (human NHE1, Figs 4 and 5) at variance with the shorter V116 and the hydrophilic S119 (Fig. 4) may contribute to form in NHE1 a hydrophobic-binding pocket more stable than the one observed in NHE5. Thus, we can speculate that the hydrophobic

character of NHE1-binding pocket, stronger than its counterpart in NHE5, may be responsible of more stable binding interactions with the hydrophobic moiety (the isopropyl – aromatic portion) of cariporide. This hypothesis is consistent with evidence from the work by Jinadasa *et al.* (2014) that, in AP-1 (NHE-deficient) cells, stably transfected with NHE1, after acid load with NH_4Cl , the intracellular pH recover was completely blocked at 1–10 μM concentration of cariporide while substantially higher concentrations of cariporide (500–1000 μM) were required to block pH_i recovery in AP-1 NHE5-expressing cells. Taken together, all the above considerations suggest that, in our study, the component of Na^+ -dependent H^+ efflux was more likely due to NHE1 activity rather than activity from other isoforms.

It has been long accepted that pH_i is a vital regulator of spermatozoa motility (Darszon *et al.* 2007, Lishko & Kirichok 2010, Fuster & Alexander 2014). Because NHE is directly responsible for transporting H^+ outside the cell in exchange for extracellular Na^+ , its inhibition is supposed to induce proton accumulation inside the cell, thus potentially leading to the inhibition of sperm motility; we therefore tested the role of NHE on the motility of sperm cells via cariporide-dependent NHE1 inhibition. Obtained data acts as an evidence, for the first time, that the NHE1 isoform may play a role in the regulation of sperm motility in a large mammalian animal model. Indeed, Woo *et al.* (2002), by using ouabain, the inhibitor of Na^+/K^+ -ATPase, questionably claimed the specific involvement of NHE1 in rat sperm motility. To our knowledge, no studies have been reported to date of a direct involvement of NHE1 in sperm motility by using its specific inhibitors. The present work represents a step forward in understanding the involvement of NHE in sperm motility, as by using a selective inhibitor, we suggest for the first time that the isoenzyme 1 of the Na^+/H^+ exchanger may affect a major motility parameter, such as total motility, and secondary kinetics parameters, such as VAP, VCL, VSL and ALH, some of them (VCL, VSL and ALH) being considered as indicators of sperm hyperactivated motility and capacitation.

The addition of cariporide had no effect on oocyte fertilization. This result could be explained considering that, despite the significant inhibitory effect on total motility and velocity parameters, a certain percentage of sperm cells may have retained normal motility, thus ensuring normal fertilization *in vitro*. Possible additional explanations may derive from the adopted swim-up and IVF procedures. In fact, during IVF, cumulus–oocyte complexes and sperm cells are co-cultured *in vitro*. Hence, we did not add cariporide in IVF medium, limiting inhibitor sperm exposure to the swim-up step, as it might have also altered pH regulation of the cumulus–oocyte complex. This would not have allowed to discriminate whether it exerted its inhibitory

action on sperm or on the oocyte or both cell types. Moreover, during IVF, bicarbonate may have activated compensatory mechanisms of pH regulation in sperm, mediated by other membrane transporters (Freitas *et al.* 2017, Pereira *et al.* 2017). Another possible explanation could be the prolonged exposure time during IVF culture, in which cariporide may have progressively lost its inhibitory effect.

An *in vivo* study (Bell *et al.* 1999) described NHE1 knockout mice as fertile, although they were reported to be extremely fragile, with most of them dying before reaching adulthood. Though comparison between *in vitro* and *in vivo* studies is not straightforward, the results of our *in vitro* assays are in agreement with those of Bell *et al.* (1999), as we did not find correlation between the NHE1-dependent motility drop and fertilization rate. Moreover, it must be pointed out that upregulation of other proteins likely involved in the same biological pathway, e.g., NHE5 (Woo *et al.* 2002), sNHE, namely NHE10 (Wang *et al.* 2003), and mtsNHE (Liu *et al.* 2010), that can compensate for the loss of NHE1 activity in NHE1-null spermatozoa to maintain sperm motility and male fertility, may have played a role in explaining the sperm fertilizing ability, as reported by Bell *et al.* (1999) and in our IVF experiments.

In conclusion, this is the first study suggesting a possible functional involvement of NHE1 in sperm intracellular pH and motility regulation using NHE1 selective inhibition. Future production of recombinant cell lines, each expressing only single NHE isoforms, is therefore envisageable as they may represent a suitable model to screen for novel isoform-specific NHE inhibitors/activators enabling dissection of the relative contribution by different NHE isoforms in sperm cell function.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-17-0456>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by Fondazione Cassa di Risparmio di Puglia (<http://www.fondazionecrpuglia.it/>). N A M was granted by the project ONEV MIUR PONa3 00134-n.254/R&C 18/05/2011 and the project GR-2011-02351396 (Ministry of Health, Young Researchers Grant 2011/2012).

Acknowledgements

The authors dedicate this work to the memory of Prof. Dario Cianci, eminent researcher in the field of livestock science, for its long-standing role in promoting interdisciplinary interactions and for having inspired a generation of scientists through his personality. Authors would like to acknowledge Dr Maria Teresa Ferrara for contributing in data collection.

References

- Baldi E, Luconi M, Bonaccorsi L & Forti G 2002 Signal transduction pathways in human spermatozoa. *Journal of Reproductive Immunology* **53** 121–131. ([https://doi.org/10.1016/S0165-0378\(01\)00089-4](https://doi.org/10.1016/S0165-0378(01)00089-4))
- Bell SM, Schreiner CM, Schultheis PJ, Miller ML, Evans RL, Vorhees CV, Shull GE & Scott WJ 1999 Targeted disruption of the murine Nhe1 locus induces ataxia, growth retardation, and seizures. *American Journal of Physiology* **276** C788–C795. (<https://doi.org/10.1152/ajpcell.1999.276.4.C788>)
- Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 248–254. ([https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3))
- Carraro-Lacroix LR, Ramirez MA, Zorn TM, Reboucas NA & Malnic G 2006 Increased NHE1 expression is associated with serum deprivation-induced differentiation in immortalized rat proximal tubule cells. *American Journal of Physiology: Renal Physiology* **291** F129–F139. (<https://doi.org/10.1152/ajprenal.00290.2005>)
- Cox JF, Alfaro V, Montenegro V & Rodríguez-Martínez H 2006 Computer-assisted analysis of sperm motion in goats and its relationship with sperm migration in cervical mucus. *Theriogenology* **66** 860–867. (<https://doi.org/10.1016/j.theriogenology.2006.01.062>)
- Cuello F, Snabaitis AK, Cohen MS, Taunton J & Avkiran M 2007 Evidence for direct regulation of myocardial Na⁺/H⁺ exchanger isoform 1 phosphorylation and activity by 90-kDa ribosomal S6 kinase (RSK): effects of the novel and specific RSK inhibitor fmk on responses to alpha1-adrenergic stimulation. *Molecular Pharmacology* **71** 799–806. (<https://doi.org/10.1124/mol.106.029900>)
- Darszon A, Trevino CL, Wood C, Galindo B, Rodríguez-Miranda E, Acevedo JJ, Hernandez-Gonzalez EO, Beltran C, Martinez-Lopez P & Nishigaki T 2007 Ion channels in sperm motility and capacitation. *Society of Reproduction and Fertility Supplement* **65** 229–244.
- Donowitz M, Ming Tse C & Fuster D 2013 SLC9/NHE gene family, a plasma membrane and organellar family of Na⁺/H⁺ exchangers. *Molecular Aspects of Medicine* **34** 236–251. (<https://doi.org/10.1016/j.mam.2012.05.001>)
- Favia M, Mancini MT, Bezzerri V, Guerra L, Laselva O, Abbattiscianni AC, Debellis L, Reshkin SJ, Gambari R, Cabrini G et al. 2014 Trimethylangelicin promotes the functional rescue of mutant F508del CFTR protein in cystic fibrosis airway cells. *American Journal of Physiology: Lung Cellular and Molecular Physiology* **307** L48–L61. (<https://doi.org/10.1152/ajplung.00305.2013>)
- Freitas MJ, Vijayaraghavan S & Fardilha M 2017 Signaling mechanisms in mammalian sperm motility. *Biology of Reproduction* **96** 2–12.
- Fuster DG & Alexander RT 2014 Traditional and emerging roles for the SLC9 Na⁺/H⁺ exchangers. *Pflügers Archiv* **466** 61–76. (<https://doi.org/10.1007/s00424-013-1408-8>)
- Goswami P, Paulino C, Hizlan D, Vonck J, Yildiz O & Kuhlbrandt W 2011 Structure of the archaeal Na⁺/H⁺ antiporter NhaP1 and functional role of transmembrane helix 1. *EMBO Journal* **30** 439–449. (<https://doi.org/10.1038/emboj.2010.321>)
- Jinadasa T, Szabo EZ, Numat M & Orlowski J 2014 Activation of AMP-activated protein kinase regulates hippocampal neuronal pH by recruiting Na⁺/H⁺ exchanger NHE5 to the cell surface. *Journal of Biological Chemistry* **289** 20879–20897. (<https://doi.org/10.1074/jbc.M114.555284>)
- Lee HC & Garbers DL 1986 Modulation of the voltage-sensitive Na⁺/H⁺ exchange in sea urchin spermatozoa through membrane potential changes induced by the egg peptide speract. *Journal of Biological Chemistry* **261** 16026–16032.
- Leung GP, Tse CM, Chew SB & Wong PY 2001 Expression of multiple Na⁺/H⁺ exchanger isoforms in cultured epithelial cells from rat efferent duct and cauda epididymidis. *Biology of Reproduction* **64** 482–490. (<https://doi.org/10.1095/biolreprod64.2.482>)
- Lishko PV & Kirichok Y 2010 The role of Hv1 and CatSper channels in sperm activation. *Journal of Physiology* **588** 4667–4672. (<https://doi.org/10.1113/jphysiol.2010.194142>)
- Liu T, Huang JC, Zuo WL, Lu CL, Chen M, Zhang XS, Li YC, Cai H, Zhou WL, Hu ZY et al. 2010 A novel testis-specific Na⁺/H⁺ exchanger is involved in sperm motility and fertility. *Frontiers in Bioscience* **2** 566–581. (<https://doi.org/10.2741/e115>)
- Mannowetz N, Wandernoth P & Wennemuth G 2012 Basigin interacts with both MCT1 and MCT2 in murine spermatozoa. *Journal of Cellular Physiology* **227** 2154–2162. (<https://doi.org/10.1002/jcp.22949>)
- Martino NA, Ariu F, Bebbere D, Uranio MF, Chirico A, Marzano G, Sardanelli AM, Cardinali A, Minervini F, Bogliolo L et al. 2016 Supplementation with nanomolar concentrations of verbascoside during in vitro maturation improves embryo development by protecting the oocyte against oxidative stress: a large animal model study. *Reproductive Toxicology* **65** 204–211. (<https://doi.org/10.1016/j.reprotox.2016.08.004>)
- Martins AD, Bernardino RL, Neuhaus-Oliveira A, Sousa M, Sa R, Alves MG & Oliveira PF 2014 Physiology of Na⁺/H⁺ exchangers in the male reproductive tract: relevance for male fertility. *Biology of Reproduction* **91** 11. (<https://doi.org/10.1095/biolreprod.114.118331>)
- Masereel B, Pochet L & Laeckmann D 2003 An overview of inhibitors of Na⁺/H⁺ exchanger. *European Journal of Medicinal Chemistry* **38** 547–554. ([https://doi.org/10.1016/S0223-5234\(03\)00100-4](https://doi.org/10.1016/S0223-5234(03)00100-4))
- Mihaila RG 2015 A minireview on NHE1 inhibitors. A rediscovered hope in oncology. *Biomedical Papers of the Faculty of Medicine of Palacký University, Olomouc, Czech Republic* **159** 519–526. (<https://doi.org/10.5507/bp.2015.060>)
- Montrose MH, Friedrich T & Murer H 1987 Measurements of intracellular pH in single LLC-PK1 cells: recovery from an acid load via basolateral Na⁺/H⁺ exchange. *Journal of Membrane Biology* **97** 63–78. (<https://doi.org/10.1007/BF01869615>)
- Mortimer ST & Maxwell WM 1999 Kinematic definition of ram sperm hyperactivation. *Reproduction, Fertility and Development* **11** 25–30. (<https://doi.org/10.1071/RD99019>)
- Najafi A, Daghigh-Kia H, Dodaran HV, Mehdipour M & Alvarez-Rodríguez M 2017 Ethylene glycol, but not DMSO, could replace glycerol inclusion in soybean lecithin-based extenders in ram sperm cryopreservation. *Animal Reproduction Science* **177** 35–41. (<https://doi.org/10.1016/j.anireprosci.2016.12.004>)
- Nejsum LN, Praetorius J & Nielsen S 2005 NKCC1 and NHE1 are abundantly expressed in the basolateral plasma membrane of secretory coil cells in rat, mouse, and human sweat glands. *American Journal of Physiology: Cell Physiology* **289** C333–C340. (<https://doi.org/10.1152/ajpcell.00228.2004>)
- Ng KYB, Mingels R, Morgan H, Macklon N & Cheong Y 2017 In vivo oxygen, temperature and pH dynamics in the female reproductive tract and their importance in human conception: a systematic review. *Human Reproduction Update* **24** 15–34. (<https://doi.org/10.1093/humupd/dmx028>)
- Oberheide K, Puchkov D & Jentsch TJ 2017 Loss of the Na⁺/H⁺ exchanger NHE8 causes male infertility in mice by disrupting acrosome formation. *Journal of Biological Chemistry* **292** 10845–10854. (<https://doi.org/10.1074/jbc.M117.784108>)
- Padan E & Landau M 2016 Sodium-proton (Na⁺/H⁺) antiporters: properties and roles in health and disease. *Metal Ions in Life Sciences* **16** 391–458. (https://doi.org/10.1007/978-3-319-21756-7_12)
- Palacin I, Vicente-Fiel S, Santolaria P & Yaniz JL 2013 Standardization of CASA sperm motility assessment in ram. *Small Ruminant Research* **112** 128–135. (<https://doi.org/10.1016/j.smallrumres.2012.12.014>)
- Paoli D, Gallo M, Rizzo F, Baldi E, Francavilla S, Lenzi A, Lombardo F & Gandini L 2011 Mitochondrial membrane potential profile and its correlation with increasing sperm motility. *Fertility and Sterility* **95** 2315–2319. (<https://doi.org/10.1016/j.fertnstert.2011.03.059>)
- Pedersen SF & Cala PM 2004 Comparative biology of the ubiquitous Na⁺/H⁺ exchanger, NHE1: lessons from erythrocytes. *Journal of Experimental Zoology Part A Comparative Experimental Biology* **301** 569–578. (<https://doi.org/10.1002/jez.a.47>)

- Pereira R, Sa R, Barros A & Sousa M 2017 Major regulatory mechanisms involved in sperm motility. *Asian Journal of Andrology* **19** 5–14. (<https://doi.org/10.4103/1008-682X.167716>)
- Perez LJ, Valcarcel A, delas Heras MA, Moses DF & Baldassarre H 1996 In vitro capacitation and induction of acrosomal exocytosis in ram spermatozoa as assessed by the chlortetracycline assay. *Theriogenology* **45** 1037–1046. ([https://doi.org/10.1016/0093-691X\(96\)00031-3](https://doi.org/10.1016/0093-691X(96)00031-3))
- Pierri CL, Parisi G & Porcelli V 2010 Computational approaches for protein function prediction: a combined strategy from multiple sequence alignment to molecular docking-based virtual screening. *Biochimica et Biophysica Acta* **1804** 1695–1712. (<https://doi.org/10.1016/j.bbapap.2010.04.008>)
- Pierri CL, Bossis F, Punzi G, De Grassi A, Cetrone M, Parisi G & Tricarico D 2016 Molecular modeling of antibodies for the treatment of TNF α -related immunological diseases. *Pharmacology Research and Perspectives* **4** e00197. (<https://doi.org/10.1002/prp2.197>)
- Rimmer SJ 1999 Demonstration of a Na⁽⁺⁾/H⁽⁺⁾ exchanger NHE1 in fresh bovine corneal endothelial cell basolateral plasma membrane. *Biochimica et Biophysica Acta* **1419** 283–288. ([https://doi.org/10.1016/S0005-2736\(99\)00076-0](https://doi.org/10.1016/S0005-2736(99)00076-0))
- Roussa E, Alper SL & Thevenod F 2001 Immunolocalization of anion exchanger AE2, Na⁽⁺⁾/H⁽⁺⁾ exchangers NHE1 and NHE4, and vacuolar type H⁽⁺⁾-ATPase in rat pancreas. *Journal of Histochemistry and Cytochemistry* **49** 463–474. (<https://doi.org/10.1177/002215540104900406>)
- Sakkas D, Ramalingam M, Garrido N & Barratt CL 2015 Sperm selection in natural conception: what can we learn from Mother Nature to improve assisted reproduction outcomes? *Human Reproduction Update* **21** 711–726. (<https://doi.org/10.1093/humupd/dmv042>)
- Siyanov V & Baltz JM 2013 NHE1 is the sodium-hydrogen exchanger active in acute intracellular pH regulation in preimplantation mouse embryos. *Biology of Reproduction* **88** 157. (<https://doi.org/10.1095/biolreprod.113.109033>)
- Slepkov ER, Rainey JK, Li X, Liu Y, Cheng FJ, Lindhout DA, Sykes BD & Fliegel L 2005 Structural and functional characterization of transmembrane segment IV of the NHE1 isoform of the Na⁽⁺⁾/H⁽⁺⁾ exchanger. *Journal of Biological Chemistry* **280** 17863–17872. (<https://doi.org/10.1074/jbc.M409608200>)
- Slepkov ER, Rainey JK, Sykes BD & Fliegel L 2007 Structural and functional analysis of the Na⁽⁺⁾/H⁽⁺⁾ exchanger. *Biochemical Journal* **401** 623–633. (<https://doi.org/10.1042/BJ20061062>)
- Sutovsky P, Hewitson L, Simerly C & Schatten G 1996 Molecular medical approaches for alleviating infertility and understanding assisted reproductive technologies. *Proceedings of the Association of American Physicians* **108** 432–443.
- Thomas JA, Buchsbaum RN, Zimniak A & Racker E 1979 Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry* **18** 2210–2218. (<https://doi.org/10.1021/bi00578a012>)
- Todisco S, Di Noia MA, Onofrio A, Parisi G, Punzi G, Redavid G, De Grassi A & Pierri CL 2016 Identification of new highly selective inhibitors of the human ADP/ATP carriers by molecular docking and in vitro transport assays. *Biochemical Pharmacology* **100** 112–132. (<https://doi.org/10.1016/j.bcp.2015.11.019>)
- Wang D, King SM, Quill TA, Doolittle LK & Garbers DL 2003 A new sperm-specific Na⁽⁺⁾/H⁽⁺⁾ exchanger required for sperm motility and fertility. *Nature Cell Biology* **5** 1117–1122. (<https://doi.org/10.1038/ncb1072>)
- Wang D, Hu J, Bobulescu IA, Quill TA, McLeroy P, Moe OW & Garbers DL 2007 A sperm-specific Na⁽⁺⁾/H⁽⁺⁾ exchanger (sNHE) is critical for expression and in vivo bicarbonate regulation of the soluble adenylyl cyclase (sAC). *PNAS* **104** 9325–9330. (<https://doi.org/10.1073/pnas.0611296104>)
- Wohlert D, Kuhlbrandt W & Yildiz O 2014 Structure and substrate ion binding in the sodium/proton antiporter PaNhaP. *Elife* **3** e03579. (<https://doi.org/10.7554/eLife.03579>)
- Woo AL, James PF & Lingrel JB 2002 Roles of the Na,K-ATPase α 4 isoform and the Na⁽⁺⁾/H⁽⁺⁾ exchanger in sperm motility. *Molecular Reproduction and Development* **62** 348–356. (<https://doi.org/10.1002/mrd.90002>)
- Yang X, Chen J, Bai H, Tao K, Zhou Q, Hou H & Hu D 2013 Inhibition of Na⁽⁺⁾/H⁽⁺⁾ exchanger 1 by cariporide reduces burn-induced intestinal barrier breakdown. *Burns* **39** 1557–1564. (<https://doi.org/10.1016/j.burns.2013.04.007>)
- Zhang Z, Yang Y, Wu H, Zhang H, Zhang H, Mao J, Liu D, Zhao L, Lin H, Tang W *et al.* 2017 Sodium-Hydrogen-Exchanger expression in human sperm and its relationship with semen parameters. *Journal of Assisted Reproduction and Genetics* **34** 795–801. (<https://doi.org/10.1007/s10815-017-0898-2>)

Received 25 July 2017

First decision 30 August 2017

Revised manuscript received 21 February 2018

Accepted 27 February 2018