

1 **Title:** Na<sup>+</sup>/K<sup>+</sup>ATPase as a signaling molecule during bovine sperm capacitation<sup>1</sup>

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3 **Short Title:** NaK-ATPase and bovine sperm capacitation

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5 **Summary sentence:** Na<sup>+</sup>/K<sup>+</sup>ATPase is involved in the regulation of protein tyrosine  
6 phosphorylation and capacitation of bovine spermatozoa

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8 **Authors:** Jacob C. Thundathil, Muhammad Anzar and Mary M. Buhr

9 Department of Animal & Poultry Science, University of Guelph, Ontario, Canada, N1G 2W1

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11 **Correspondence and reprint requests:**

12 Dr. Mary M. Buhr  
13 Professor and Associate Dean (Academic)  
14 Ontario Agricultural College  
15 University of Guelph  
16 Guelph, Ontario N1G 2W1  
17 Tel: (519) 824-4120 ext 53492  
18 fax: (519) 824-0870  
19 Email: mbuhr@uoguelph.ca

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27 **Abstract**

28  $\text{Na}^+/\text{K}^+$ ATPase is a heteromeric, integral membrane protein composed of two  
29 polypeptides, alpha and beta, and found to be active in many cell types including testis and  
30 spermatozoa.  $\text{Na}^+/\text{K}^+$ ATPase is a well-known ion transporter, but ouabain (a specific inhibitor of  
31  $\text{Na}^+/\text{K}^+$ ATPase) binding to the  $\text{Na}^+/\text{K}^+$ ATPase in somatic cells initiates responses that are similar  
32 to signaling events associated with bovine sperm capacitation. The objectives of this study were  
33 to demonstrate the presence of  $\text{Na}^+/\text{K}^+$ ATPase in bovine sperm and investigate its role in the  
34 regulation of bovine sperm capacitation. The presence of  $\text{Na}^+/\text{K}^+$ ATPase in sperm from mature  
35 Holstein bulls was demonstrated by immunoblotting and immunocytochemistry using a  
36 monoclonal antibody developed in mouse against the beta 1 polypeptide of  $\text{Na}^+/\text{K}^+$ ATPase.  
37 Ouabain binding to the  $\text{Na}^+/\text{K}^+$ ATPase inhibited motility (decreased progressive motility,  
38 average path velocity, and curvilinear velocity), induced tyrosine phosphorylation and  
39 capacitation, but did not increase intracellular calcium levels in spermatozoa. Furthermore,  
40 ouabain binding to the  $\text{Na}^+/\text{K}^+$ ATPase induced depolarization of sperm plasma membrane.  
41 Therefore, ouabain binding to the  $\text{Na}^+/\text{K}^+$ ATPase induced sperm capacitation through  
42 depolarization of sperm plasma membrane and signaling via the tyrosine phosphorylation  
43 pathway without an appreciable increase in intracellular calcium. To our knowledge this is the  
44 first report on the signaling role of  $\text{Na}^+/\text{K}^+$ ATPase in mammalian sperm capacitation.

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## 50 **Introduction**

51           After ejaculation, mammalian spermatozoa reside in the female reproductive tract for a  
52 species dependent period of time to attain fertilizing ability, during which they undergo a series  
53 of structural and functional modifications collectively known as capacitation [1-4]. Although  
54 capacitation is a prerequisite for fertilization, the role of individual sperm proteins in the  
55 capacitation process is still under investigation. Understanding the specific role of individual  
56 sperm proteins in capacitation and fertilization process is important for fertility investigations  
57 and improving the success of assisted reproductive technologies.

58            $\text{Na}^+/\text{K}^+$ ATPase, a heteromeric integral membrane protein, belongs to the family of P-type  
59 ATPases, composed of two polypeptides, alpha (110 kDa) and the glycosylated beta (55 kDa),  
60 and found to be active in many cell types including testis and spermatozoa [5, 6]. The alpha  
61 polypeptide is the catalytic unit of the enzyme, containing the binding sites for cations, cardiac  
62 glycosides and ATP. Four alpha polypeptide isoforms have been identified (alpha 1, alpha 2,  
63 alpha 3 and alpha 4), which are specific to different cell types and appear to regulate different  
64 functions [7]. The alpha 1 and testis-specific alpha 4 isoform of  $\text{Na}^+/\text{K}^+$ ATPase (ATP1A1 and  
65 ATP1A4, respectively) have been isolated from the testis, and are localized in spermatozoa [5,  
66 8]. The inhibition of the activity of ATP1A4, isoform has been shown to eliminate sperm  
67 motility [5]. The beta polypeptide is involved in the maturation of the enzyme, localization to the  
68 plasma membrane and stabilization of the  $\text{K}^+$  - occluded intermediate form of the protein [5].  
69 Besides its role as an energy transducing ion pump involved in the active extrusion of  $\text{Na}^+$  from  
70 the nerve cell [9],  $\text{Na}^+/\text{K}^+$ ATPase also act as a sodium pump for the coupled active transports of  
71  $\text{Na}^+$  and  $\text{K}^+$  across the plasma membrane of animal cells [10, 11]. This enzymatic activity results  
72 in the production of an electrochemical gradient that is required for many cellular processes,

73 including maintenance of the resting membrane potential, regulation of the osmotic balance and  
74 generation of the  $\text{Na}^+$  gradient that is necessary for the transport of many ions and other  
75 substrates across plasma membrane [12, 13]. The chemical gradient established by the  
76  $\text{Na}^+/\text{K}^+$ ATPase is important for the restoration of low intracellular  $\text{Ca}^{2+}$  concentrations after  
77 contraction of the cardiac muscle. Cardiac glycosides such as ouabain and digoxin can inhibit the  
78 activity of  $\text{Na}^+/\text{K}^+$ ATPase, which increases intracellular  $\text{Ca}^{2+}$  concentration and cardiac  
79 contractility [14]. Inhibition of the  $\text{Na}^+/\text{K}^+$ ATPase disrupts cellular chemical gradients so that  
80 there is a drop in intracellular  $\text{K}^+$  levels with a concomitant increase in  $\text{Na}^+$  levels [13]. Since the  
81  $\text{Na}^+/\text{Ca}^{2+}$  exchanger uses this gradient to pump out  $\text{Ca}^{2+}$  from the cell, dampening this gradient  
82 acts as a rate limiting step for the movement of  $\text{Ca}^{2+}$  out of the cell via  $\text{Na}^+/\text{Ca}^{2+}$  exchanger [7].

83         It has been recently shown that in cardiac myocytes and several other cell types,  
84  $\text{Na}^+/\text{K}^+$ ATPase acts as a signal transducer in addition to its effect on ion transport [15].  
85 Interaction of the endogenous cardiac glycoside ouabain [16, 17] with  $\text{Na}^+/\text{K}^+$ -ATPase increases  
86 the production of reactive oxygen species (ROS) and tyrosine phosphorylation in several proteins  
87 through a mechanism independent of changes in intracellular concentrations of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  in  
88 cardiac myocytes [18] and epithelial cell lines [19]. The most proximal effects of ouabain  
89 binding of  $\text{Na}^+/\text{K}^+$ ATPase are the Src activation, epidermal growth factor receptor (EGFR)  
90 transactivation, activation of protein kinase C (PKC) and extracellular signal-regulated kinase  
91 (ERK) family of mitogen activated protein kinase pathway (MAPK) [15]. Ouabain binding of  
92  $\text{Na}^+/\text{K}^+$ ATPase causes transcriptional regulation of several genes associated with cardiac  
93 hypertrophy through multiple signal transduction pathways including MAPK [20].

94         In brief, studies on somatic cells suggest that interaction of ouabain with  $\text{Na}^+/\text{K}^+$ ATPase  
95 initiates a series of cellular responses, which are similar to the molecular events associated with

96 mammalian sperm capacitation. For example, increase in intracellular  $\text{Na}^+$  and  $\text{Ca}^{2+}$  [3], tyrosine  
97 phosphorylation in a cohort of sperm proteins [21-27], generation of reactive oxygen species [4],  
98 activation of ERK family of MAPK [28-30] are important events associated with sperm  
99 capacitation. Furthermore, Fraser et al. [31] observed that incubation of mouse sperm in presence  
100 of ouabain increased the rate of capacitation in mouse spermatozoa and we have previously  
101 demonstrated  $\text{Na}^+/\text{K}^+$ ATPase activity from the head plasma membrane extracted from bovine  
102 spermatozoa [32]. Based on these observations, we hypothesized that  $\text{Na}^+/\text{K}^+$ -ATPase signaling  
103 is involved in the regulation of bovine sperm capacitation.

104 This study aimed to localize  $\text{Na}^+/\text{K}^+$ ATPase in bull spermatozoa and to investigate the  
105 role of this enzyme in the regulation of sperm motility, plasma membrane potential, tyrosine  
106 phosphorylation and capacitation in bovine spermatozoa. To our knowledge, this is the first report  
107 on the role of  $\text{Na}^+/\text{K}^+$ ATPase in the regulation of bovine sperm capacitation.

108

## 109 **Materials and Methods**

### 110 *Materials*

111 The following reagents were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON):  
112 *Pisum sativum* agglutinin conjugated to fluorescein isothiocyanate (FITC-PSA), 3-isobutyl-1-  
113 methylxanthine (IBMX),  $\text{N}^6, 2'$ - O-dibutyryl cAMP (dbcAMP), lysophosphatidylcholine (LPC),  
114 H89 (N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide), dithiothreitol (DTT),  
115 heparin sodium salt (from porcine intestinal mucosa, 180 USP units/mg), BSA (fraction V,  
116 catalog number A-4503), paraformaldehyde 95%, luminol 97% HPLC grade, p-Coumaric acid  
117 and Indo-1/AM (4-(6-Carboxy-2-indolyl)-4'-methyl-2,2'-(ethylenedioxy) dianiline N, N, N', N'-  
118 tetra acetic acid tetrakis (acetoxymethyl) ester), Dimethyl sulfoxide, Pluronic acid and ouabain.

119 Propidium Iodide and DiBac<sub>4</sub> (3) were purchased from Molecular Probes (Eugene, OR). Percoll  
120 was obtained from Amersham Pharmacia Biotech (Baie d'Urfé, QC, Canada). PVDF membrane  
121 (pore size, 0.45 µm) was purchased from Millipore Corporation (Bedford, MA). Anti  
122 phosphotyrosine monoclonal antibody (clone 4G10) developed in mouse, goat antimouse IgG  
123 conjugated with horse radish peroxidase (HRP), rat kidney microsomal preparation containing  
124 Na<sup>+</sup>/K<sup>+</sup>ATPase beta 1 polypeptide (ATP1B1 previously known as Atpb or Atpb-1) in  
125 homogenized buffer (positive control), anti- ATP1B1 (mouse monoclonal IgG), goat anti-rabbit  
126 IgG-FITC conjugated (goat polyclonal IgG), goat anti-mouse IgG-FITC conjugated (goat  
127 polyclonal IgG), normal mouse IgG, normal rabbit IgG were purchased from Upstate  
128 Biotechnology (Lake Placid, NY). 30% Acrylamide/Bis solution 29:1, 10x Tris/Glycine/SDS  
129 buffer, Precision plus protein kaleidoscope molecular mass standards, N, N, N', N'-Tetra-methyl  
130 ethylenediamine (TEMED), Ammonium persulfate were purchased from Bio-Rad Laboratories  
131 (Mississauga, ON, Canada). Ponceau Red, Glycine and Sodium vanadate were purchased from  
132 Fisher Scientific (Far Lawn, NJ). Kodak Scientific imaging film X-OMAT LS was obtained  
133 from Mandel Scientific (Guelph ON, Canada). Kodak GBX developer and replenisher, and  
134 Kodak GBX fixer and replenisher were purchased from Medtec (Mississauga, ON, Canada).  
135

136 *Immunodetection and immunolocalization of Na<sup>+</sup>/K<sup>+</sup>ATPase in bovine spermatozoa*

137 A monoclonal antibody developed in mouse against ATP1B1 polypeptide of  
138 Na<sup>+</sup>/K<sup>+</sup>ATPase was used to detect ATP1B1 in bovine sperm. Head plasma membrane of bovine  
139 sperm was prepared as described previously [32]. Protein extracts prepared from the head plasma  
140 membrane were used for SDS-PAGE and immunoblotting as described below. Rat brain extract  
141 provided by the manufacturer was used as the positive control.

142 For immunolocalization of ATP1B1, ejaculated bull spermatozoa ( $500 \mu\text{l}$ ,  $15 \times 10^6/\text{ml}$   
143 washed in phosphate buffered saline (PBS; 125 mM NaCl, 8 mM  $\text{Na}_2\text{HPO}_4$ , 2 mM  
144  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 5 mM KCl and 5 mM Dextrose; pH adjusted to 7.4) were attached to poly-l-  
145 lysine coated coverslips. Spermatozoa on coverslips were either fixed with 1% paraformaldehyde  
146 in PBS for 10 min at room temperature or permeabilised with cold ( $-20 \text{ }^\circ\text{C}$ ) ethanol for 30 sec.  
147 Spermatozoa were washed in PBS (2 x 5 min). For immunostaining, spermatozoa were incubated  
148 in PBS containing 10% normal goat serum (blocking buffer) for 30 min at room temperature  
149 followed by mouse anti-rabbit ATP1B1 clone (1:20 diluted in blocking buffer) for 1h at  $37 \text{ }^\circ\text{C}$ .  
150 Following two five minute washes in PBS, spermatozoa were incubated with FITC-conjugated  
151 goat anti-mouse IgG (1: 50 in blocking buffer) for 1h at  $37 \text{ }^\circ\text{C}$ . For control, spermatozoa were  
152 either incubated in blocking buffer or in mouse IgG instead of primary antibody.  
153

#### 154 *Preparation of incubation media, percoll gradients and test reagents*

155 Modified Tyrodes bicarbonate-buffered medium (Sp-TALP) and modified Tyrode's  
156 Hepes-buffered medium (Sp-TALPH) were prepared as described previously [33, 34]. 90%  
157 percoll (isoosmotic, 280 mOsm) was prepared by mixing 100 % percoll with 10X Sp-TALPH at  
158 a ratio of 9:1(v/v) and 45% percoll was prepared by mixing isotonic percoll as prepared above  
159 with equal volume of Sp-TALPH (1X) as described by [35]. Working solutions of ouabain and  
160 heparin were prepared in complete Sp-TALP medium [34] on the day of use. Stock solutions of  
161 IBMX (10 mM in Dimethyl sulfoxide) and dbc AMP (100 mM in milliQ water) were prepared  
162 and stored at  $-20 \text{ }^\circ\text{C}$ . On the day of use the working solution of these reagents were prepared by  
163 mixing these stock solutions (1:1).

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169 *Preparation of sperm for motility assessment, tyrosine phosphorylation and capacitation*

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171 Fresh semen ejaculates collected from mature Holstein-Friesian bulls were provided by  
172 the Genetic Corporation (Gencor) Inc., Guelph, ON. Semen samples with at least 60-65%  
173 motility were used for the study. Semen samples were immediately diluted 1:5 with Sp-TALPH  
174 and transported to the laboratory within 30 min after collection in thermos maintained at 35 °C.  
175 Semen samples were subjected to percoll wash on two-layer percoll gradients (45-90%) by  
176 centrifugation (700g, 30 min, 25°C; 35). The resulting sperm pellet was resuspended in Sp-  
177 TALPH and washed to remove percoll (380g, 10 min, 25°C). The sperm pellet was resuspended  
178 in Sp-TALP and washed again to remove Sp-TALPH (380 g, 10 min, 25°C). The concentration  
179 of the resulting sperm pellet was determined using a hemocytometer and adjusted to  $100 \times 10^6$   
180 cells/ml with Sp-TALP.

181

182 *Evaluation of the effect of inhibition of  $\text{Na}^+/\text{K}^+$  ATPase on sperm motility, tyrosine*

183 *phosphorylation and capacitation*

184 Semen ejaculates obtained from different bulls ( $n = 3$ ) were used for the evaluation of  
185 motility, capacitation and tyrosine phosphorylation. The sperm pellet prepared as above from  
186 each ejaculate was split into series of 1.5 ml, eppendorf tubes and concurrently used for the  
187 evaluation of motility, tyrosine phosphorylation and capacitation.

188 To study the effect of  $\text{Na}^+/\text{K}^+$  ATPase on motility, sperm samples ( $50 \times 10^6/\text{ml}$ ) were  
189 incubated (39°C, 5%  $\text{CO}_2$ , high humidity) with either 0, 1, 10, 100 or 1000  $\mu\text{M}$  ouabain for 5 h.  
190 At 0, 2 and 5h of incubation, 10  $\mu\text{l}$  of sperm preparation was drawn from each treatment group  
191 and adjusted to a final concentration of  $5 \times 10^6$  sperm/ml in egg yolk-Tris extender at 35°C [36],  
192 to avoid sperm sticking to the microscopic slides, for motility analysis. Eight  $\mu\text{L}$  of semen was  
193 loaded onto a glass microscope slide, covered with a coverslip, and analyzed at 35°C with the

194 Bovine Motility Program on a Hamilton-Thorne motility analyzer (Beverly, MA) for % motility  
195 and % progressive motility with the following analysis set up; frame rate: 60/sec; frames  
196 acquired: 50 per sec; minimum contrast: 55 pixels; minimum cell size: 7 pixels; threshold  
197 straightness: 80 microns/sec; medium VAP cut-off: 100 microns/sec; low VAP cut-off: 20  
198 microns/sec; static size limits: 0.60-2.99 pixels; static intensity limits: 0.59-1.41 pixels; static  
199 elongation limits: 0-73%; non motile head size: 10; Non-motile head intensity 101 and slow  
200 cells, which have a speed less than the above state VAP cut-off, are labeled as static or not  
201 moving.

202 For the evaluation of tyrosine phosphorylation and capacitation, sperm preparations were  
203 incubated (39°C, 5% CO<sub>2</sub>, high humidity, 5h) with or without heparin (10 µg per ml), a  
204 combination of dbcAMP (1 mM) + IBMX (0.1mM) or ouabain (1 µM, 10 µM, 100 µM or 1  
205 mM). Each sample preparation for treatments contained 5 x 10<sup>6</sup> sperm in 100 µl volume. For  
206 capacitation experiments, 2 aliquots of sperm preparations were maintained per treatment group  
207 to evaluate capacitation status at time 0 and 5h of incubation. At each time point, sperm samples  
208 were incubated with either LPC (100 µg per ml) or Sp-TALPH for 30 min in an incubator at  
209 39°C with 5% CO<sub>2</sub> and under high humidity. The acrosomal status of spermatozoa (200  
210 spermatozoa per slide) was determined using fluorescein isothiocyanate-conjugated *Pisum*  
211 *sativum* agglutinin as described previously [24, 34]. The proportion of spermatozoa undergoing  
212 acrosome reaction was determined for each treatment group treated with LPC and corrected for  
213 spontaneous acrosome reaction by subtracting the proportion of acrosome reacted spermatozoa  
214 present in parallel samples incubated in Sp-TALPH alone.

215  
216 For the evaluation of tyrosine phosphorylation, two aliquots of sperm preparations were  
217 maintained per treatment group and sperm were processed at 0 and 5h of incubation as described  
218 previously [34, 37] with the following modifications. Sperm were concentrated (10,000 g, 3 min,  
219 room temperature) and the was pellet washed (10,000 g for 5 min) in 1 ml PBS containing 0.2

220 mM Na<sub>2</sub>VO<sub>3</sub>. The pellet was resuspended in 50 µl of PBS containing 0.2 mM Na<sub>2</sub>VO<sub>3</sub> and 12.5  
221 µl of 5X sample buffer (containing DTT and SDS). The preparation was mixed well and boiled  
222 for 5 min at 100°C, centrifuged (10,000g for 5 min) and the supernatants were used for SDS-  
223 PAGE and immunoblotting.

224 For SDS-PAGE and immunoblotting, sperm extracts prepared as above were subjected to  
225 electrophoresis on 10% polyacrylamide gels for 15 min at 75 V for stacking followed by  
226 electrophoresis at 100 V until the running front reached the bottom of the gel at room  
227 temperature. The gels were equilibrated in transfer buffer for 30 min at 4 °C and  
228 electrotransferred to Immobilon P using Tris- Glycine buffer, pH 8.5, containing 20% methanol.  
229 The membranes were incubated with a solution of skim milk (5%, w/v) in Tris (20 mM, pH 7.8)-  
230 buffered saline containing Tween 20 (0.1%, v/v; TTBS, 1h) and then treated with a monoclonal  
231 antibody developed in mouse against phospho-tyrosine proteins (1:10,000 in TTBS  
232 supplemented with 0.1% (w/v) sodium azide overnight at 4°C. After washing with TTBS,  
233 membranes were incubated with goat anti-mouse IgG conjugated with horseradish peroxidase for  
234 45 min at 20°C and washed again with TTBS. Positive immunoreactive bands were detected  
235 using enhanced chemiluminescence detection system. At the end of the experiments, blots were  
236 rinsed in distilled water and Ponceau stained [34] to ascertain that the amount of proteins loaded  
237 in each well was the same. The specificity of the anti-phosphotyrosine-antibody (clone 4G10)  
238 was determined as recommended by the manufacturers; the antibody recognized tyrosine  
239 phospho proteins from the EGF stimulated cell lysate (positive control provided by the  
240 manufacturers). Parallel blots were incubated with secondary antibody alone to determine non-  
241 specific bands.

242

243 *Effects of inhibition of Na<sup>+</sup>/K<sup>+</sup>ATPase on intracellular calcium levels during incubation under*  
244 *capacitating conditions*

245 Percoll washed sperm samples (n = 4) were loaded with a ratiometric calcium fluorescent  
246 probe Indo-1AM as described previously [38]. These sperm samples were incubated in  
247 capacitating conditions (39°C with 5% CO<sub>2</sub> and under high humidity) with Sp-TALP, heparin or  
248 ouabain in presence of 2 mM calcium and evaluated for intracellular calcium at 0h and after 4h  
249 of incubation in capacitating conditions using a flow cytometer (Beckman-Coulter EPICS Elite,  
250 Hialeah, FL). Immediately prior to flow cytometric analysis, 500 µl of sperm samples (2 x 10<sup>6</sup>  
251 sperm /ml) from each treatment were removed and propidium iodide (PI) was added to a final  
252 concentration of 20 µM. The fluorescence intensities were recorded for a minimum of 10,000  
253 spermatozoa at three wavelengths: for PI, conditions were: excitation at 488 nm and emission at  
254 610 nm. The fluorescent probe Indo-1AM was excited with ultraviolet light (325 nm) and  
255 fluorescence emissions were recorded for each sperm at two wavelengths; 381 ± 5 nm (Indo-1  
256 AM bound to calcium) and 525 ± 5 nm (Indo unbound). Data were subsequently analysed by  
257 FCS Express (De Novo Software, Thornhill, ON, Canada) to calculate relative intracellular  
258 calcium concentration of live cells. Cells positive for PI (dead) were first excluded, and the  
259 relative intracellular calcium of the remaining PI negative cells were plotted based on the ratio of  
260 fluorescence at the two Indo wavelengths (381 ± 5 nm/ 525 ± 5 nm; Indo bound /unbound to  
261 calcium) for each individual sperm cell. This created two discrete populations, and the number of  
262 sperm in each population were counted and expressed as a percentage of the total live cells  
263 analysed and mean percentages were calculated based on 4 replicates conducted with ejaculates  
264 from different bulls.

265  
266 *Effect of inhibition of Na<sup>+</sup>/K<sup>+</sup>ATPase on membrane potential of spermatozoa incubated under*  
267 *capacitating conditions*

268 Effect of inhibition of  $\text{Na}^+/\text{K}^+$ ATPase on intracellular calcium level as described above  
269 and membrane potential were done using sperm preparations from the same ejaculate and these  
270 experiments were replicated 4 times using ejaculates from different bulls. Percoll washed  
271 spermatozoa were incubated with Sp-TALP alone, heparin (10  $\mu\text{g}$ ) or ouabain (100  $\mu\text{M}$ ) for 4h  
272 under capacitating conditions and the effect of inhibition of  $\text{Na}^+/\text{K}^+$ ATPase on sperm membrane  
273 was measured by flow cytometry (Beckman-Coulter EPICS Elite, Hialeah, FL) using DiBac<sub>4</sub> (3)  
274 as the fluorescent probe. DiBac<sub>4</sub> (3) is an anionic lipophilic potential –sensitive dye with  
275 excitation at 488 nm and emission at 525 nm. With membrane depolarization more dye enters  
276 the cytosol and the intensity of emitted fluorescence increases; with hyperpolarization  
277 fluorescence decreases [39]. Stock solution of DiBac<sub>4</sub> (3) was prepared in DMSO according to  
278 the manufacturer's instructions. At time 0 and 4 h of incubation, 25  $\mu\text{M}$  DiBac<sub>4</sub> (3) (based on the  
279 results of preliminary experiments) and 20  $\mu\text{M}$  PI were added to sperm aliquots from each  
280 treatment group and the preparations were incubated at 37°C for 10 min and a minimum of  
281 10,000 events were evaluated by flow cytometry. The dead population of spermatozoa was  
282 excluded from analysis based on the uptake of propidium iodide. The DiBac<sub>4</sub> (3) fluorescence of  
283 spermatozoa was plotted, and the mean intensity of fluorescence and the mean proportion of live  
284 cells for each treatment group were derived using the FCS Express software.  
285

286 *Requirement of extracellular calcium for tyrosine phosphorylation and capacitation induced by*  
287 *ouabain*

288 To assess the need for exogenous calcium, sperm samples from the same ejaculate (n=3)  
289 were evaluated for tyrosine phosphorylation and capacitation as described above, except that  
290 Percoll gradients and Sp-TALPH were prepared without adding calcium and the incubation of  
291 spermatozoa was done in calcium containing (2 mM  $\text{CaCl}_2$ ) or calcium deficient (no  $\text{CaCl}_2$   
292 added) Sp-TALP.

293

## 294 **Statistical analysis**

295 Analysis of variance (two-tailed; unpaired values) was used to compare the effects of  
296 treatments on motility, capacitation, intracellular calcium levels and membrane potential.  
297 Comparison of means between treatment groups was then done by the protected least-significant  
298 difference test. A difference was considered statistically significant when  $P < 0.05$ .

299

## 300 **Results**

301

### 302 *Immunodetection and immunolocalization of $\text{Na}^+/\text{K}^+$ ATPase in bovine spermatozoa*

303 A monoclonal antibody developed in mouse against beta 1 polypeptide of  $\text{Na}^+/\text{K}^+$  ATPase  
304 recognized four bands (90, 60, 45 and 40 kDa) from an extract of proteins prepared from sperm  
305 head plasma membrane and the antibody recognized 2 bands (45 and 40 kDa) from the control  
306 rat brain extract (Fig. 1). Incubation of parallel membranes with secondary antibody alone did  
307 not elicit any signal (data not shown). Immunostaining demonstrated a uniform distribution of  
308  $\text{Na}^+/\text{K}^+$  ATPase on acrosomal, post-acrosomal and mid-piece regions of fixed (non-  
309 permeabilised) spermatozoa (Fig. 2A). However, the fluorescence pattern on the acrosomal and  
310 post-acrosomal regions of permeabilised spermatozoa changed to irregular clusters (patchy) (Fig.  
311 2B). Incubation of sperm preparations with FITC-conjugated secondary antibody or non immune  
312 mouse IgG alone did not elicit any fluorescent signal (data not shown).

313

### 314 *Effect of inhibition of $\text{Na}^+/\text{K}^+$ ATPase on sperm motility*

315 Inhibition of  $\text{Na}^+/\text{K}^+$  ATPase with ouabain did not affect the total % of motile sperm (Fig.  
316 3A), which remained high throughout the incubation period. Ouabain reduced the percentage of  
317 progressively motile sperm, VAP and VCL of spermatozoa in a dose dependent manner after  
318 incubation in capacitating conditions over a period of 5 hours (Fig. 3B, C and D, respectively),  
319 but had no apparent effect on the ALH of spermatozoa (Fig. 3E). Head-to-head agglutination

320 was noted in a proportion of spermatozoa incubated with ouabain, and were excluded from  
321 evaluation based on size.  
322

323 *Effect of inhibition of Na<sup>+</sup>/K<sup>+</sup> ATPase on tyrosine phosphorylation and capacitation*

324 Incubation of spermatozoa in the presence of ouabain induced tyrosine phosphorylation  
325 in a cohort of sperm proteins (11, 50, 80, 100, 130, 200, 250 kDa). The patterns of tyrosine  
326 phosphorylation for the samples that were incubated with ouabain were similar to that induced  
327 by a combination of dbcAMP + IBMX (Fig. 4A) and these protein bands appeared to be more  
328 intense for sperm treated with ouabain compared to those treated with heparin (Fig. 4A). The  
329 intensity of the tyrosine phosphorylation in these proteins appeared to increase with increased  
330 amounts of ouabain. When parallel samples were assessed for capacitation (sensitivity to LPC-  
331 induced acrosome reaction), ouabain-induced capacitation occurred concurrently with tyrosine  
332 phosphorylation in spermatozoa. Incubation of sperm preparations with 10 μM, 100 μM or 1  
333 mM ouabain significantly increased the proportion of sperm undergoing acrosome reaction in  
334 response to LPC compared to sperm samples incubated in Sp-TALP alone (Fig. 4B). The  
335 proportion of sperm undergoing capacitation in response to 1mM ouabain was similar to that of  
336 dbcAMP + IBMX ( $27 \pm 7\%$  and  $28.6 \pm 1.3\%$ , respectively), whereas the effects of lower  
337 concentrations of ouabain (10 or 100 μM) were similar to that of heparin ( $17 \pm 2.5\%$ ,  $17.6 \pm$   
338  $0.5\%$ ,  $14.3 \pm 0.3\%$  for sperm exposed to 10 or 100 μM ouabain, or heparin, respectively; Fig.  
339 4B). There was no significant increase in non-LPC-induced acrosome reaction at 5h compared to  
340 0h among different treatment groups (data not shown).

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344 *Effects of inhibition of Na<sup>+</sup>/K<sup>+</sup>ATPase on intracellular calcium levels during incubation under*  
345 *capacitating conditions*

346         The proportion of dead sperm was identified based on PI uptake and excluded from  
347 analysis as shown in Fig. 5A. Incubation of spermatozoa in capacitating conditions result in 2  
348 populations of live spermatozoa (Fig. 5B), with differing amounts of intracellular calcium (high  
349 and low, Fig. 5C and D, respectively). The actual mean amount of intracellular calcium within  
350 each population was not affected by treatments. More sperm were in the low-calcium-level  
351 population regardless of treatment, and only heparin affected the proportion of sperm in each  
352 population; after 4h of incubation in the presence of heparin, fewer sperm were in the low-  
353 calcium population and more in the high-calcium population (Fig. 5E, F, \*  $P < 0.05$ ).

354  
355 *Requirement of extracellular calcium for tyrosine phosphorylation and capacitation induced by*  
356 *ouabain*

357         Although ouabain did not significantly increase the proportion of spermatozoa with  
358 higher calcium levels, incubation of spermatozoa with ouabain did increase tyrosine  
359 phosphorylation levels in sperm proteins with a concomitant increase in the proportion of  
360 spermatozoa undergoing capacitation as indicated by LPC-induced acrosome reaction (Fig. 4A  
361 and B). Therefore, we investigated the requirement of extracellular calcium for tyrosine  
362 phosphorylation and capacitation in bovine sperm induced by ouabain. The intensity of tyrosine  
363 phosphorylation increased in a cohort of sperm proteins (50, 60, 80, 100, 130 kDa) for the  
364 groups treated with heparin, dbc AMP+IBMX or ouabain regardless of the level of extracellular  
365 calcium levels after 5h of incubation in capacitating conditions compared to spermatozoa  
366 incubated in Sp-TALP alone (Fig. 6A). In general, higher intensity of tyrosine phosphorylation  
367 was observed in sperm samples incubated with ouabain including increased tyrosine  
368

369 phosphorylation in two additional protein bands at 200 and 250 kDa (Fig. 6A). Interestingly,  
370 sperm samples incubated with heparin, dbcAMP + IBMX or ouabain demonstrated that an 11  
371 kDa protein underwent tyrosine phosphorylation only in presence of extracellular calcium and  
372 the intensity of this protein band was higher for the sperm samples incubated in presence of  
373 dbcAMP + IBMX or ouabain. Capacitation assay demonstrated that extracellular calcium was  
374 required for heparin or dbcAMP+ IBMX induced capacitation based on acrosome reaction data  
375 (Fig. 6B) but not for the ouabain-induced increase in the proportion of sperm undergoing  
376 acrosome reaction, suggesting that extracellular calcium is not mandatory for the ouabain-  
377 induced acrosome reaction.

378

379 *Inhibition of Na<sup>+</sup>/K<sup>+</sup>ATPase depolarizes sperm plasma membrane*

380 Incubation of spermatozoa in presence of ouabain increased DiBac<sub>4</sub> (3) fluorescence  
381 indicating that inhibition of Na<sup>+</sup>/K<sup>+</sup>ATPase depolarized the plasma membrane of living  
382 spermatozoa (Fig. 7, A-C). Heparin did not alter Dibac<sub>4</sub> (3) fluorescence. The proportion of live  
383 spermatozoa did not differ among treatment groups during the period of incubation (Fig. 7D).

384

## 385 **Discussion**

386

387 In addition to the well-known activity of Na<sup>+</sup>/K<sup>+</sup>ATPase as an ion transporter involved in  
388 capacitation-related depolarization of the sperm membrane, we demonstrate here for the first  
389 time that this enzyme has characteristics of a signaling molecule responsible for tyrosine  
390 phosphorylation in the earliest stages of capacitation. In addition, we clarify the location of the  
391 enzyme on the sperm head, and its mechanism of action: the enzyme must be inactivated to  
392 induce membrane depolarization and the signaling cascade that are hallmarks of capacitation

393 induction. The signaling cascade appears to be independent of calcium, although we have  
394 identified at least one low molecular mass protein whose tyrosine phosphorylation requires  
395 incubation in capacitating conditions in the presence of dbcAMP + IBMX or ouabain and is  
396 greatly enhanced by calcium.

397

398 *Immunodetection and immunolocalization of Na<sup>+</sup>/K<sup>+</sup>ATPase in bovine sperm*

399 A monoclonal antibody identified the ATP1B1 in four protein bands (90, 60, 45 and 40  
400 kDa) in the proteins of head plasma membrane from bull sperm. The molecular masses of two  
401 protein bands (45 and 40 kDa) were similar to those present in the positive control (rat brain  
402 extract) indicating the presence of ATP1B1 in bovine sperm. Lack of specific signal from control  
403 membrane i.e., incubated with secondary antibody alone confirmed the specificity of monoclonal  
404 antibody to recognize ATP1B1 in bull spermatozoa and positive control. However, the antibody  
405 also recognized two additional protein bands (90 and 60 kDa) in the head plasma membrane of  
406 bull sperm, which may represent total or partial dimerization of ATP1B1 or ATP1B1 with  
407 different levels of glycosylation. The beta polypeptides are heavily glycosylated and  
408 glycosylation patterns of different isoforms of beta polypeptides may differ between species  
409 [12]. Moreover, organ-specific glycoforms of Na<sup>+</sup>/K<sup>+</sup>ATPase have also been reported previously  
410 [40, 41]. Our immunolocalization study suggests that Na<sup>+</sup>/K<sup>+</sup>ATPase is uniformly distributed in  
411 the plasma membrane of sperm head (acrosomal and post-acrosomal regions) and midpiece of  
412 fixed non-permeabilised spermatozoa (Fig. 2A). The change in fluorescence pattern in  
413 permeabilised spermatozoa (Fig. 2B) suggests that Na<sup>+</sup>/K<sup>+</sup>ATPase is also present intracellularly  
414 or on the outer acrosomal membrane. Rat epididymal sperm possesses both ATP1A1 and  
415 ATP1A4 localized to the midpiece of the flagellum [5]. In human sperm, ATP1A4 is localized to

416 the principal piece of flagellum. In the present study, the localization of  $\text{Na}^+/\text{K}^+$ ATPase over the  
417 midpiece region indicates its possible role in the regulation of bull sperm motility [5]. These  
418 differences among species in the location of  $\text{Na}^+/\text{K}^+$ ATPase suggest different roles of this protein  
419 in sperm physiology [42].

420  
421  *$\text{Na}^+/\text{K}^+$ ATPase affects sperm motility*

422  
423 Ouabain, a specific inhibitor of  $\text{Na}^+/\text{K}^+$ ATPase [16-17, 20], reduced the percentage of  
424 progressively motile sperm and the velocity at which ejaculated bull spermatozoa moved, in a  
425 dose-and time-dependent manner. When  $10\mu\text{M}$  ouabain inhibited the *Atp1a4* alone in freshly  
426 isolated epididymal rat sperm, it reduced the total percent of motile sperm to the same level as  
427 when  $10\text{ mM}$  ouabain inhibited all of the  $\text{Na}^+/\text{K}^+$ ATPase [5]. Here, with ejaculated bovine  
428 sperm,  $100\mu\text{M}$  or  $1\text{ mM}$  ouabain reduced the percentage of progressively motile but not the  
429 percentage of total motile spermatozoa. These differences in results between bull and rat  
430 spermatozoa [5], may be due to the differences in species (rat vs. bull) and sources of  
431 spermatozoa (epididymal vs. ejaculated). It has been reported that ouabain at  $1\text{mM}$  concentration  
432 inhibited progressive motility of bovine sperm within 30 min [43]. In our study, a Hamilton-  
433 Thorn motility analyzer was used for motility evaluation which detected a statistically  
434 significant reduction in progressive motility after 5h of incubation under capacitating conditions  
435 and thus confirming [43]  $\text{Na}^+/\text{K}^+$ ATPase's impact on sperm motility parameters.

436  
437 There was no evident change in ALH, which is contrary to the expectation that ALH  
438 increases with capacitation. However, many sperm underwent head- to- head agglutination  
439 during incubation in the presence of ouabain, preventing the measurement of their motility  
440 characteristics. Agglutination of spermatozoa during incubation in presence of known  
441 capacitating agents has been reported previously [33]. Therefore, agglutination of spermatozoa

441 during incubation in presence of ouabain suggests that ouabain binding to the  $\text{Na}^+/\text{K}^+$ ATPase  
442 induces head plasma membrane modifications associated with capacitation.

443

444  *$\text{Na}^+/\text{K}^+$ ATPase affects tyrosine phosphorylation and capacitation*

445

446 A role of cAMP/PKA dependent protein tyrosine phosphorylation during sperm  
447 capacitation has been reported from several species [21-27]. However, the specific plasma  
448 membrane proteins involved in the regulation of protein tyrosine phosphorylation remain to be  
449 identified. Recent studies on somatic cells demonstrated that inhibition of  $\text{Na}^+/\text{K}^+$ ATPase  
450 induces tyrosine phosphorylation in proteins [19]. Furthermore, Fraser et al. [31] observed that  
451 incubation of mouse epididymal sperm with ouabain increased the rate of capacitation and we  
452 have previously demonstrated  $\text{Na}^+/\text{K}^+$ ATPase activity in the head plasma membrane of bovine  
453 spermatozoa [32]. Now we show that ouabain induces tyrosine phosphorylation in ejaculated  
454 spermatozoa in a dose dependent manner. In general, the pattern of ouabain-induced tyrosine  
455 phosphorylation was similar, albeit more intense, than that of dbcAMP + IBMX (a  
456 phosphodiesterase inhibitor) in bovine sperm as has been shown previously [27, 34]. We also  
457 show a cohort of sperm proteins with higher molecular masses (130, 200 and 250 kDa) and a low  
458 molecular mass protein (11 kDa) appeared to be undergoing tyrosine phosphorylation.

459 Ouabain also induced capacitation (measured as the ability to undergo LPC-induced  
460 acrosomal exocytosis) in a dose dependent manner suggesting that  $\text{Na}^+/\text{K}^+$ ATPase inhibition-  
461 associated increase in tyrosine phosphorylation occurs in synchrony with capacitation. Although  
462 ability to fertilize an oocyte remains as the best indicator of capacitation status [3], the  
463 association between tyrosine phosphorylation, and LPC-induced acrosome reaction links the

464 ability of sperm membranes to fuse, with the molecular events associated with capacitation in  
465 bovine [24, 27, 34] and human [26, 44] spermatozoa.

466

467 *Inhibition of Na<sup>+</sup>/K<sup>+</sup>ATPase did not influence intracellular calcium levels in spermatozoa*

468 Increase in intracellular calcium levels is one of the major events occurring during sperm

469 capacitation in several species [4, 26]. Na<sup>+</sup>/K<sup>+</sup>ATPase is an energy-transducing ion pump in

470 most mammalian cells, actively transporting Na<sup>+</sup> and K<sup>+</sup> across the plasma membrane.

471 Inhibition of Na<sup>+</sup>/K<sup>+</sup>ATPase can lead into a small increase in intracellular Na<sup>+</sup> concentration,

472 which can in turn increase intracellular Ca<sup>2+</sup> concentration through the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger [45,

473 46]. While the proportion of live spermatozoa did not differ among treatment groups, incubation

474 of sperm with heparin increased the proportion of spermatozoa with higher intracellular calcium

475 levels. However, incubation with ouabain for 4 hours did not significantly increase the

476 proportion of spermatozoa with higher calcium levels. Therefore, Na<sup>+</sup>/K<sup>+</sup>ATPase inhibition leads

477 to tyrosine phosphorylation and sperm capacitation without increasing intracellular calcium

478 levels, similar to the calcium independent signaling mechanisms leading to the activation of

479 MAPK, tyrosine phosphorylation and generation of ROS following ouabain inhibition of

480 Na<sup>+</sup>/K<sup>+</sup>ATPase in somatic cells [47].

481

482

483 *Requirement of extracellular calcium for tyrosine phosphorylation and capacitation induced by*  
484 *heparin and ouabain*

485 Since ouabain did not increase intracellular calcium concentration, we tested the calcium-

486 dependence of the ouabain-induced tyrosine phosphorylation and capacitation. All of the proteins

487 but one were tyrosine phosphorylated equally with or without 2mM calcium when sperm were

488 incubated with heparin, dbcAMP + IBMX or ouabain, suggesting that extracellular calcium

489 levels did not influence the level of tyrosine phosphorylation in spermatozoa. The requirement of

490 extracellular calcium for tyrosine phosphorylation is controversial and still under investigation

491 [20]. Previous studies have demonstrated that an increase in tyrosine phosphorylation could be

492 achieved in mouse [21] and human [48] sperm by increasing the extracellular concentration of  
493  $\text{Ca}^{2+}$ . Contrary to these observations, a recent study demonstrated that the presence of calcium in  
494 the external medium decreases tyrosine phosphorylation in both human and mouse spermatozoa  
495 [49] by decreasing the availability of intracellular ATP. Similarly, an inhibitory effect of  
496 extracellular calcium on tyrosine phosphorylation in a 55 kDa protein was reported from  
497 epididymal bovine sperm [50]. Therefore the predominant calcium-independence of tyrosine  
498 phosphorylation in the current study is not inconsistent with other published results. The  
499 significance of calcium-dependent tyrosine phosphorylation of the 11 kD protein observed in this  
500 study remains to be elucidated.

501 Calcium was required for both heparin and dbcAMP+IBMX -induced acrosomal  
502 exocytosis, as expected given the well-established requirement of extracellular  $\text{Ca}^{2+}$  for  
503 mammalian sperm capacitation and acrosome reaction [51-54]. However, ouabain-induced  
504 capacitation occurred in calcium deficient medium, suggesting that plasma membrane  
505 modifications in spermatozoa induced by  $\text{Na}^+/\text{K}^+$ ATPase inhibition were sufficient to support  
506 LPC-stimulated acrosomal exocytosis in bovine sperm. Also, the incubation medium was  
507 calcium deficient but not  $\text{Ca}^{2+}$  free and therefore extracellular  $\text{Ca}^{2+}$  could still be available for  
508 membrane fusion. In addition, internal stores of  $\text{Ca}^{2+}$  localized in the acrosomal vesicle [54] may  
509 be a possible source of calcium for the membrane fusion. Conversely, extracellular calcium is  
510 not an absolute requirement for acrosome reaction in response to certain agonists [55]. The  
511 difference in the calcium dependence of the heparin- and ouabain-induced capacitation suggests  
512 that ouabain and heparin induces acrosomal exocytosis through different mechanisms.

513

#### 514 *Inhibition of $\text{Na}^+/\text{K}^+$ ATPase depolarizes sperm plasma membrane*

515

516 The enzymatic activity of  $\text{Na}^+/\text{K}^+$ ATPase results in the production of an electrochemical  
517 gradient that is required for many cellular processes, including maintenance of the resting  
518 membrane potential [12]. Inhibition of the  $\text{Na}^+/\text{K}^+$ ATPase disrupts cellular chemical gradients so

519 that there is a drop in intracellular  $K^+$  levels with a concomitant increase  $Na^+$  levels, which leads  
520 to the depolarization of plasma membrane [56]. Membrane potential can couple external signals  
521 to cellular responses and this process is particularly relevant in transcriptionally inactive  
522 spermatozoa, in which many physiological processes are controlled by ion fluxes [57].  
523 Incubation of spermatozoa in the presence of ouabain for a period of 4h increased the uptake of  
524 DiBac<sub>4</sub> (3) an anionic fluorescent probe indicating that ouabain depolarizes the plasma  
525 membrane of spermatozoa; depolarization was not observed in heparin-treated sperm. This  
526 difference between heparin- and ouabain-induced capacitation further supports the suggestion  
527 that these two agents induce acrosomal exocytosis through different mechanisms. Interestingly,  
528 Zeng et al. [58] observed that epididymal mouse sperm and ejaculated bovine spermatozoa  
529 undergo hyperpolarization during incubation under capacitating conditions. These differences in  
530 results may be due to the fact that ouabain incubation would inhibit  $Na^+/K^+$ ATPase, leading to  
531 persistent depolarization of sperm plasma membrane, resulting in tyrosine phosphorylation and  
532 capacitation in spermatozoa as reported based on studies in somatic cells [19].

533         Although tyrosine phosphorylation was induced in spermatozoa incubated with ouabain,  
534 it is not clear whether spermatozoa complete the capacitation process before initiating  
535 biochemical changes associated with acrosome reaction. This is particularly relevant considering  
536 that inhibition of  $Na^+/K^+$ ATPase induces depolarization of sperm membrane, an event associated  
537 with acrosome reaction [43] and acrosomal exocytosis of spermatozoa following incubation in  
538 presence of ouabain is independent of extracellular calcium levels. Therefore, inhibition of  
539  $Na^+/K^+$ ATPase and associated tyrosine phosphorylation may “prematurely” induce acrosome  
540 reaction before sperm completes capacitation process.

541 The physiological relevance of ouabain-induced functional modification of spermatozoa  
542 remains to be elucidated. Although ouabain is not identified from reproductive tissues, its  
543 synthesis from the adrenal gland [17, 59] and its presence in blood plasma [60] suggests that  
544 spermatozoa may be exposed to this compound during its passage through the female  
545 reproductive tract with a possible physiological role in the fertilization process by inducing  
546 capacitation and/or acrosome reaction.

547 In summary, the present study demonstrated that  $\text{Na}^+/\text{K}^+$ ATPase is involved in the  
548 regulation of signaling mechanisms leading to tyrosine phosphorylation and capacitation in  
549 bovine spermatozoa, which appeared to be occurring without an appreciable increase in  
550 intracellular calcium. The level of tyrosine phosphorylation and membrane depolarization  
551 following  $\text{Na}^+/\text{K}^+$ ATPase inhibition appeared to be sufficient to support membrane fusion and  
552 acrosomal exocytosis in spermatozoa. The localization of  $\text{Na}^+/\text{K}^+$ ATPase in spermatozoa and its  
553 role in the regulation of sperm capacitation demand further studies to investigate its role in the  
554 fertilization process per se.

555

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709

710 **Figure legends**

711

712 **FIG. 1. Immunodetection of Na<sup>+</sup>/K<sup>+</sup>ATPase in bovine spermatozoa.** Immunoblotting using a  
713 mouse monoclonal antibody developed against beta 1 polypeptide of Na<sup>+</sup>/K<sup>+</sup>ATPase detected  
714 four protein bands; lane 1-3: protein extract prepared from sperm head plasma membranes of  
715 three bulls; lane 4: Positive control (rat brain extract) provided by the manufacturer.  
716 Immunoblotting of membrane using secondary antibody alone (control) did not demonstrate any  
717 specific signal (data not shown).

718

719 **FIG. 2. Immunolocalization of Na<sup>+</sup>/K<sup>+</sup>ATPase in bovine spermatozoa.** Na<sup>+</sup>/K<sup>+</sup>ATPase beta 1  
720 polypeptide was immunolocalized to sperm plasma membrane of acrosome (arrow head) and  
721 post-acrosome (short arrow) and midpiece regions (long arrow) with even distribution in fixed  
722 (intact) spermatozoa (X600; A). The distribution of Na<sup>+</sup>/K<sup>+</sup>ATPase was patchy in anterior  
723 acrosome (arrow head) and post acrosomal (short arrow) regions of permeabilised spermatozoa  
724 (X600; B). Incubation of spermatozoa with nonimmune mouse IgG or secondary antibody alone  
725 did not elicit a fluorescent signal (data not shown).

726

727 **FIG. 3. Effect of ouabain on motility parameters of bovine sperm during incubation over a**  
728 **period of 5 hours.** Sperm preparations were incubated with ouabain at different concentrations  
729 (0-1000 µM) and its effects on total motility (A) progressive motility (B), Average path velocity  
730 (VAP; C), curve linear velocity (VCL; D) and amplitude of lateral head displacement (ALH; E)  
731 of spermatozoa were evaluated using computer assisted semen evaluation. Values presented are

732 the mean  $\pm$  SEM of three independent experiments using semen samples from three bulls.  $*P <$   
733 0.05

734

735 **FIG. 4. Inhibition of Na<sup>+</sup>/K<sup>+</sup>ATPase induced tyrosine phosphorylation and capacitation in**  
736 **bovine spermatozoa.** Percoll washed sperm preparations ( $5 \times 10^6$  in 100  $\mu$ l volume) were  
737 incubated in presence of ouabain at different concentrations and the level of tyrosine  
738 phosphorylation (A) was evaluated at the beginning of incubation (0h) and at the end of the  
739 incubation (5h). Arrow heads at the right side of the immunoblot indicate protein bands showing  
740 a change in the level of phosphorylation in different treatments. Parallel sperm preparations were  
741 evaluated for capacitation status (B) at 0h and at 5h of incubation. Percentage of sperm  
742 undergoing acrosome reaction in response to LPC was considered as the percentage capacitated  
743 spermatozoa. These values were corrected by subtracting the percentage of cells undergoing  
744 spontaneous acrosome reaction from the respective groups as described in the materials and  
745 methods. The values presented in panel B are the mean  $\pm$  SEM of three independent experiments  
746 using semen samples from three bulls (abc;  $P < 0.05$ ).

747

748 **FIG. 5. Effect of ouabain on intracellular calcium levels in spermatozoa.** Sperm containing  
749 Indo-1AM, a ratiometric fluorescent calcium chelator, were incubated in SP-TALP alone,  
750 heparin 10  $\mu$ g per ml or ouabain 100  $\mu$ M. At 0 and at 4h of incubation, an aliquot of sperm from  
751 each treatment group ( $2 \times 10^6$  sperm/ml) were exposed to propidium iodide and fluorescence  
752 intensity assessed at 610 nm (PI fluorescence)  $381 \pm 5$  nm (indo bound to calcium) and  $525 \pm 5$   
753 (indo with no calcium bound). Sperm were separated on the basis of their PI fluorescence (A;  
754 R2: dead sperm) and those with low fluorescence (A; R1: live sperm) had the ratio of

755 fluorescence intensity at 381/525 nm calculated (B). This resulted in two distinct populations,  
756 and the relative intracellular calcium content (as expressed by the ratio; C and D) and the  
757 numbers of sperm in each population (E, F) were quantified. The values presented in panel E  
758 and F are mean  $\pm$  SEM of four independent experiments using semen samples from four bulls.  
759 \* $P < 0.05$ ; FS: forward scatter.

760

761 **FIG. 6. The requirement of extracellular calcium for tyrosine phosphorylation and**  
762 **capacitation in bovine spermatozoa.** Sperm preparations ( $5 \times 10^6$  in 100  $\mu$ l volume) were  
763 incubated in SP-TALP deficient in calcium and SP-TALP with 2mM calcium. Tyrosine  
764 phosphorylation (A) and capacitation were evaluated as described for Fig. 4B. The values  
765 presented in panel B are the mean  $\pm$  SEM of three independent experiments using semen samples  
766 from three bulls. ab:  $P < 0.05$ .

767

768 **FIG. 7. Effect of inhibition of  $\text{Na}^+/\text{K}^+$ ATPase on sperm membrane potential.**

769 Sperm preparations were incubated in SP-TALP alone, 100 $\mu$ M ouabain or 10  $\mu$ g heparin per ml.  
770 At 0 and at 4h of incubation, sperm samples were loaded with 25  $\mu$ M DiBac<sub>4</sub> (3) and Propidium  
771 Iodide (PI). Sperm that were PI positive (dead, A: R2) were not analysed for DiBac<sub>4</sub> (3)  
772 fluorescence. The DiBac<sub>4</sub> (3) fluorescence of live spermatozoa (A: R1) was evaluated (B). The  
773 values presented in panels C and D are mean  $\pm$  SEM of four independent experiments using  
774 semen samples from four bulls. The ouabain treated sperm at 4h incubation had significantly  
775 greater fluorescence than all others (\* $P < 0.05$ ) indicating that this population of spermatozoa  
776 had undergone membrane depolarization (C). The proportion of live spermatozoa did not differ  
777 among treatment groups (D).

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FIG. 1. Thundathil et al.

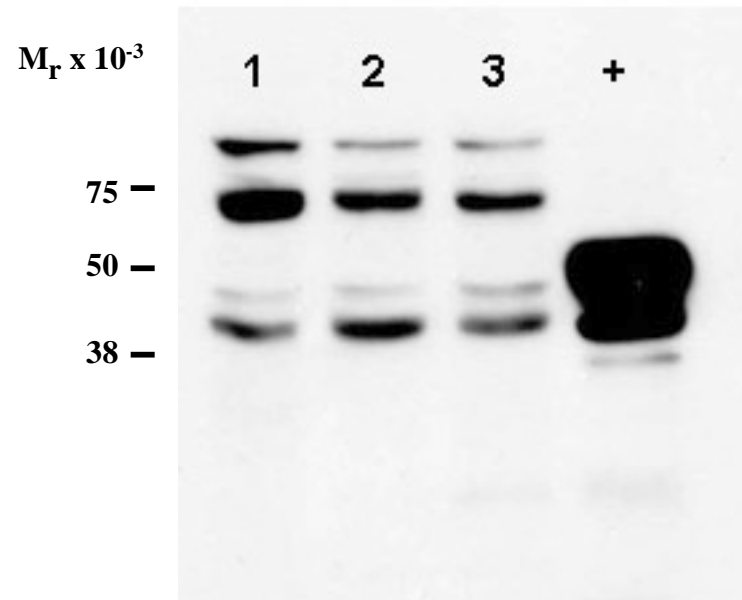
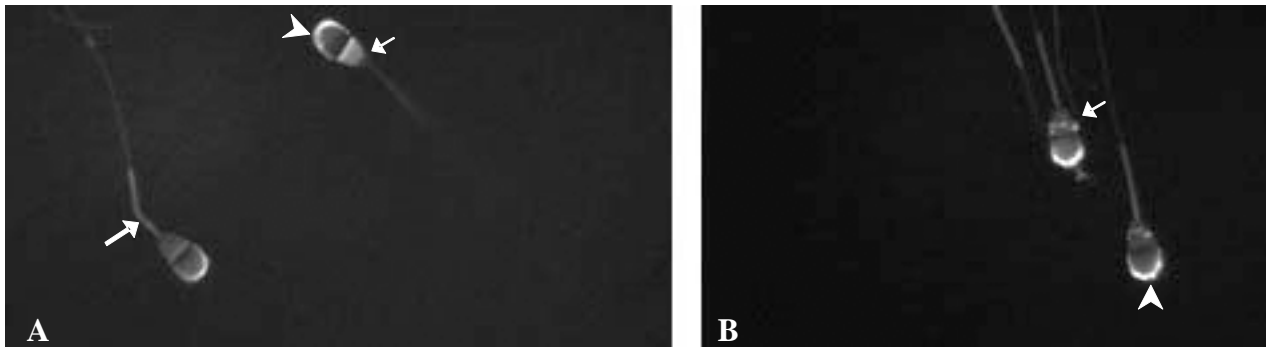


FIG. 2. Thundathil et al.



**A: Non permeabilized cells; B: Permeabilized cells**

FIG. 3.  
Thundathil et al.

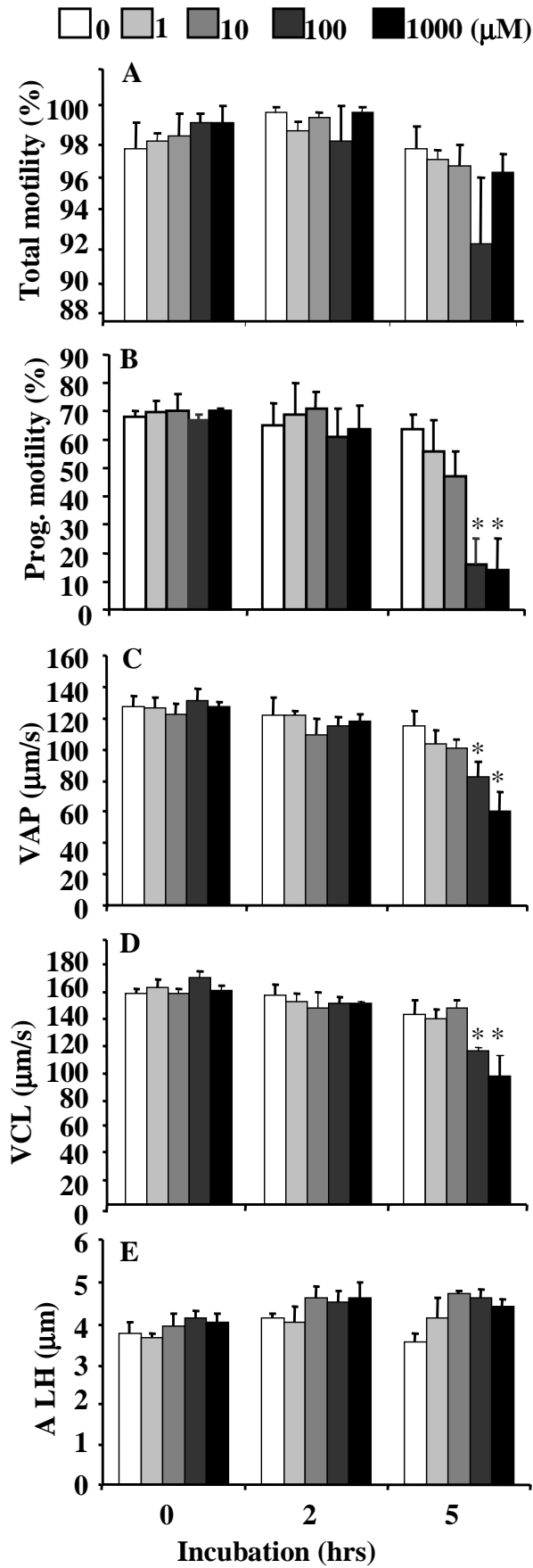


FIG. 4. Thundathil et al.

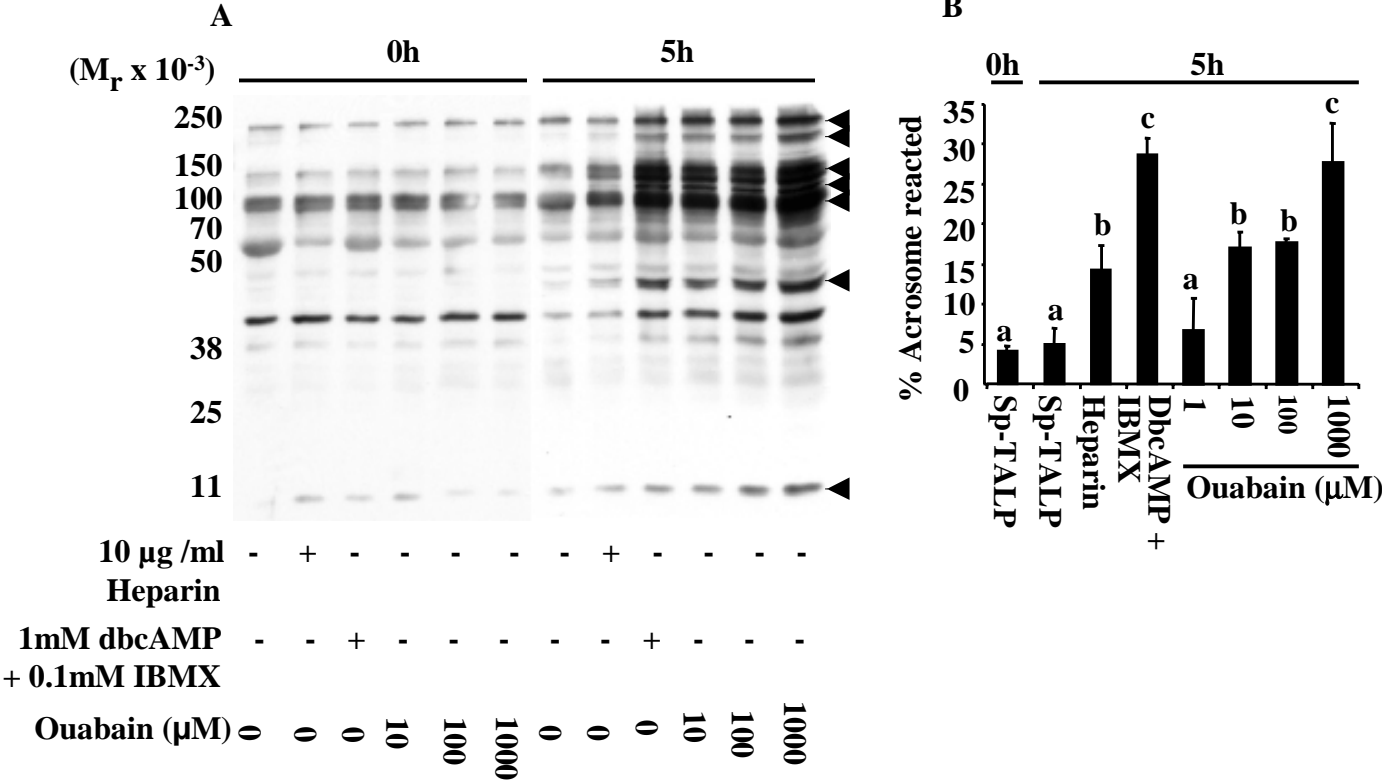


FIG.5. Thundathil et al.

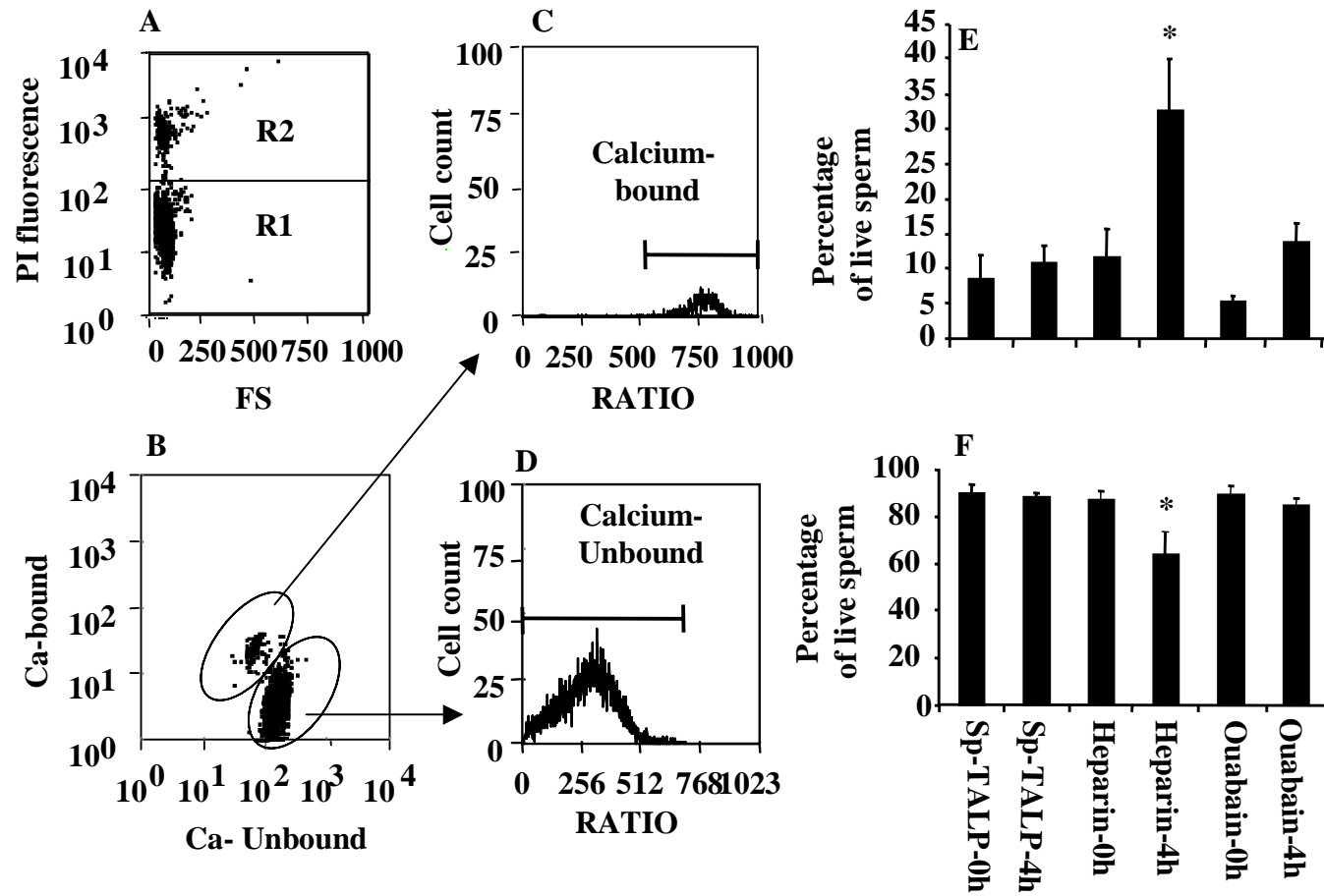


FIG. 6. Thundathil et al.

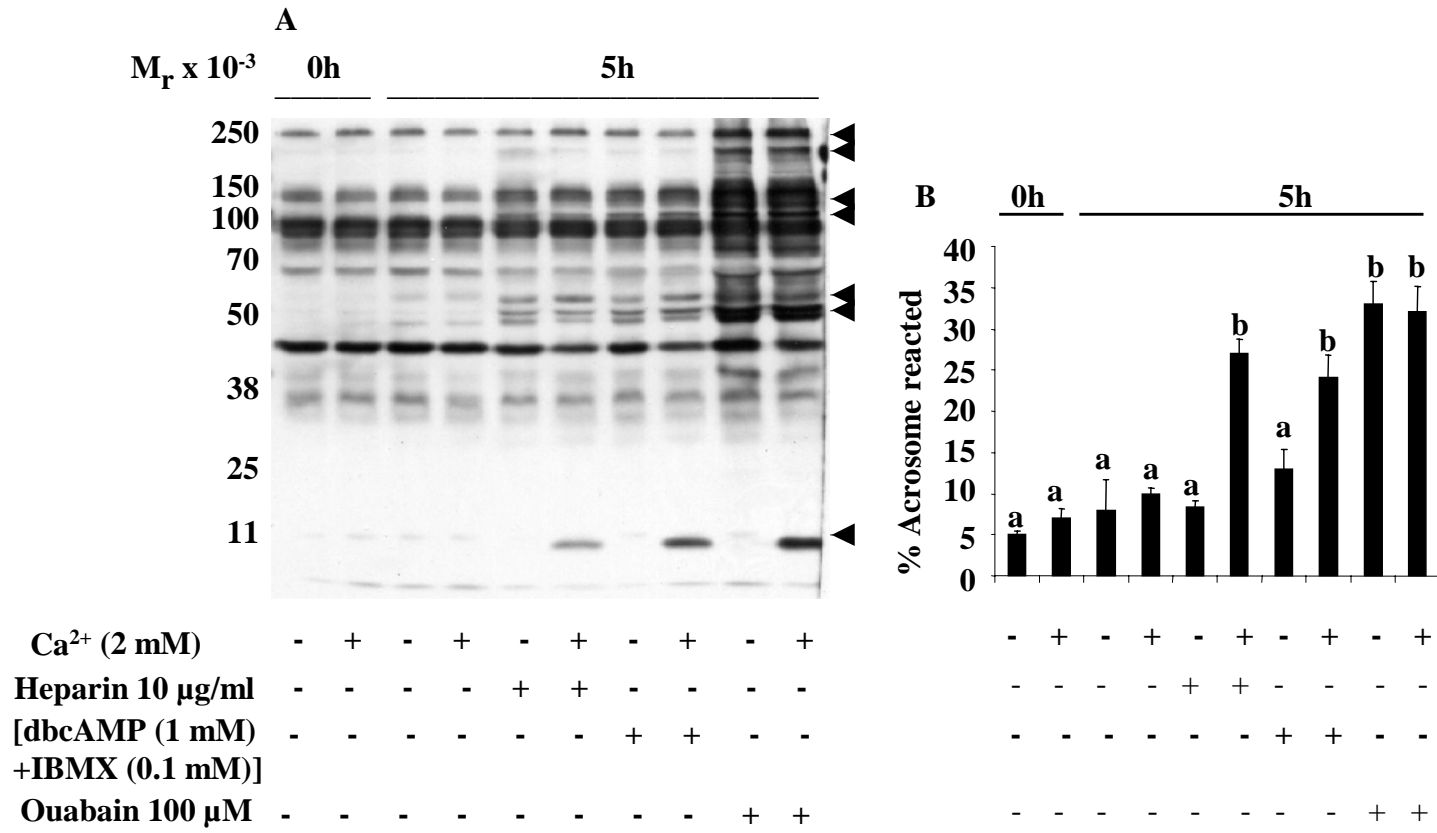


FIG.7. Thundathil et al.

