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Functional and molecular characterization of voltage gated sodium channel Na_V 1.8 in bull spermatozoa

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ABSTRACT

The aim of our study was to characterize the voltage gated sodium channel Na_V 1.8 in bull spermatozoa. Forty ejaculates were collected from four *Hariana* bulls and semen samples were pooled in view of the nonsignificant variations between different ejaculates. Functional characterization was undertaken using A-803467, a selective blocker of Na_V1.8, and veratridine as an opener of the voltage gated sodium channels while molecular characterization was done using western blotting and indirect immunofluorescence assays. *In vitro* capacitation was induced using heparin, and to study the functional involvement of Na_V 1.8 in regulation of capacitation induced hyper sperm motility, A-803467 was used. Selective blocking of Na_V 1.8 by A-803467 at 6 and 8 μM concentration significantly ($P < 0.05$) decreased the forward progressive sperm motility in a time-dependent manner, while, blocking at higher concentrations (10 and 15 μM) resulted in fast forward motility in spermatozoa after 2 h of incubation and it was observed up to 3 h. Treatment of sperm cells with veratridine (6, 8, 10, 15, 20 μM) resulted in concentration- and time-dependent increase in forward progressive sperm motility and it persisted up to 4 h. However, hyperactive motility was induced by veratridine at higher concentrations (10 and 15 μM) after 2 h of incubation. *In vitro* capacitated spermatozoa treated with A-803467 revealed significant ($P < 0.05$) reduction in forward progressive motility after 2 h of incubation. Both A-803467 and veratridine altered the percentage of spermatozoa showing high mitochondrial transmembrane potential in concentration- and time-dependent manner. High concentrations (10 and 15 μM) of A-803467 and veratridine resulted in bent neck condition in spermatozoa along with significant ($P < 0.05$) reduction in membrane integrity (HOST negative). Immunoblot revealed the presence of a single protein band of 260 kDa molecular weight along with positive immunoreactivity (IR) in head, neck, middle piece and tail of the spermatozoa. Strongest IR was observed in the neck and middle piece whereas weak IR was observed in tail and acrosomal region of the spermatozoa. Results of our present study evidently revealed the presence of voltage gated sodium channel Na_V1.8 in bull spermatozoa and its functional involvement in regulation of spermatozoa dynamics in terms of motility, membrane integrity, acrosome integrity, capacitation and mitochondrial transmembrane potential. Further studies are warranted to unravel their mechanistic pathways and/or their interaction with other ion channels in regulating sperm dynamics.

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1. Introduction

Functional regulation of spermatozoa in the microenvironments of female genital tract is very complex and involves the bidirectional crosstalk of sperm cells with female genital tract along with the fluids secretion [3]. It not only involves the fast changes within the sperm cells but also the rapid communication of sperm cells with their outer environment which ultimately enables the spermatozoa to be competent enough to result in fertilization [7]. These minute cellular regulations of sperm cell functions are mediated by ion channels present in different compartments of both the developing as well as mature sperm cells [14]. Therefore, functional and molecular characterization of ion channels will facilitate the understanding of various physiological regulatory pathways involved in both the male and female reproduction.

Molecular and electrophysiological techniques have revealed the presence of ion channels and their functional roles in mammalian spermatozoa [6,8]. Ca^{2+}, K^{+}, Cl^{-}, Na^{+}, TRP and H^{+} channels have been found to be present in the head, acrosome, neck, middle piece and flagella of sperm cells and these regulate various physiological functions in spermatozoa such as motility, viability, volume regulation, acrosome reaction, capacitation and intracellular ionic balance [6–8]. Recently, ion channels have been found to regulate fertility; and thus have opened new vistas in infertility research [3].

Na_v 1.8 channels are involved in transmission of pain signals induced by cold, heat and mechanical stimuli and are confined to dorsal root ganglia, heart and retina [1,9,17]. Recently, these have also been reported to be present in human spermatozoa and play key role in spermatozoa functions [2,11] as are primarily involved in regulation of ionic gradient across the spermatozoa membrane and maintenance of membrane potential (E_m) [4,5] and sperm motility [2]. However, absence of Na_v 1.8 channel in Na_v 1.8 null mice did not affect their fertility status [1]. Apparently no information is available regarding presence of Na_v 1.8 in spermatozoa of any species of domestic animals including bulls, and their functional role, therefore, the present study was undertaken to unravel the presence of Na_v 1.8 channels in bull spermatozoa and to elucidate their involvement in sperm dynamics.

2. Materials and methods

2.1. Chemicals and reagents

FITC- PSA, Chlortetracycline hydrochloride, Veratridine, A-803467, heparin, DAB system (Sigmafast tablets), goat anti mouse IgG-HRP, Anti mouse IgG- FITC conjugate, DABCO, HEPES were purchased from (Sigma, St Louis, USA). Primary antibody Nav 1.8 (sc 168694, Santacruz Biotechnology, USA); BSA Fraction V, PBS, total cell lysis protein isolation buffer (Amresco, USA), DMSO (Merck, Germany), protein quantification kit (Gennei, Merck, India), Mito Capture Apoptosis detection kit (Merck-Millipore, Germany) were purchased. All other chemicals for electrophoresis and western blotting were procured from Amresco, USA unless and until stated.

2.2. Collection of semen

Semen samples were collected using artificial vagina as per the standard procedure from four apparently healthy *Hariana* bulls, maintained under semi intensive system of rearing, during morning hours between 8 and 9 a.m. Total forty ejaculates were collected, ten from each bull. Immediately after collection, tubes containing semen were placed in water bath at 32–34 °C for further processing. Before evaluation, each of the ejaculate was diluted with phosphate buffer saline (PBS) of pH 7.2 having no Ca^{2+} and Mg^{2+} to achieve the final concentration of 1×10^6 sperm cells/100 μL. This concentration was maintained in all the tubes for the all experiments.

2.3. Evaluation of semen quality

Diluted semen samples were examined employing standard microscopic procedures. Evaluation of per cent forward progressive motility was carried out using phase contrast microscope having thermostatic stage [15], live per cent of spermatozoa and percentage of abnormal spermatozoa (bent neck) by using Eosin-Nigrosin staining and spermatozoa with intact plasma membrane by hypo-osmotic swelling test using 125mOsm/L hypoosmotic solution [16]. Forward progressive sperm motility was evaluated on an automated thermostatic stage maintaining temperature of 37 °C. The motility pattern of sperm samples was done as per the standard guidelines of WHO and as described earlier [11,15]. Rapid progressive motile spermatozoa were taken as "A" grade, slow progressive motile spermatozoa were taken as "B" grade, non progressive motile spermatozoa were taken as "C" grade and non motile spermatozoa were taken as "D" grade. Progressive motility (A + B), non-progressive motility (C) and immotility (D) were measured as a per cent of total (A + B + C + D) that was considered as 100%. During the study, 30 replicates were collected and (A + B) were determined for the evaluation of sperm progressive motility.

2.4. Grouping of spermatozoa for evaluation of effect of different drugs on sperm functional dynamics

Collected sperm cells were divided into different groups. For blocking of Na_v 1.8 channel, A- 308467, a selective Na_v 1.8 blocker was used. Stock solutions (1 μM) veratridine (selective agonist of Na_v) and A-308467 (selective antagonist of Nav 1.8) were prepared in DMSO and each μL of the drug solutions was containing 1 μM agonist or antagonist. To rule out the effect of DMSO, the solvent used, effect of DMSO alone was also studied. To study the effects of veratridine and A-803467 on progressive sperm motility, different groups were made viz., I-negative control (PBS), vehicle control (DMSO) as II (10 μL) and veratridine as III (10 μM) and veratridine and A-803467 as IV (veratridine-10 μM + A-803467-8 μM). To study the effects of A-803467 on capacitated spermatozoa, different groups were made viz., I-negative control (PBS), capacitated spermatozoa as II, vehicle control (DMSO) as III (8 μL), and capacitated + A-803467 as IV (Capacitated spermatozoa + A-803467-8 μM). For evaluating effect of A-803467 on sperm motility, different groups were made viz., I-negative control (PBS), vehicle control (DMSO) as II to VI (6, 8, 10, 15 & 20 μL respectively) and test groups were VII to XI (6, 8, 10, 15, and 20 μM A-803467). For evaluation effect on per cent live spermatozoa count, different groups were made viz., I-negative control (PBS), vehicle control (DMSO) as II to V (6, 8, 10, & 15 μL respectively) and test groups were VI to IX (6, 8, 10, and 15 μM A-803467). For the evaluation of effects of A-803467 on mitochondria transmembrane potential, HOST, acrosome integrity, capacitation status and sperm abnormality (bent neck condition), different groups were made viz., I-negative control (PBS), vehicle control (DMSO) as II to IV (6, 8, &10 μL respectively) and test groups were V to VII (6, 8, and 10 μM A-803467).

2.5. In vitro induction of capacitation

In vitro induction of capacitation in spermatozoa was carried as per the method for buffalo spermatozoa with little modifications [13]. Briefly, after three washings of the semen samples with sperm Tyrode's medium for spermatozoa (TALP) with 10 mM bicarbonate and 40 mM HEPES at 500 × g for 5 min each, the final sperm pellet