Immunolocalization of Phospholipase C Zeta 1 in Water Buffalo Sperm and Its Role in Oocyte Activation

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Abstract: The present study aimed to investigate the expression and localization of Phospholipase C zeta 1 (PLCZ1) in water buffalo sperm and determine its localization pattern following the induction of early fertilization events. Initially, water buffalo semen under uncapacitated conditions underwent western blot analysis to detect and localize the PLCZ1 protein via immunofluorescence, utilizing antibodies specific to buffalo PLCZ1. In a parallel experiment, a subset of sperm underwent *in-vitro* capacitation in a medium containing bicarbonate, followed by treatment with a calcium ionophore to induce the acrosome reaction, providing evidence of capacitation. The findings revealed the detection of PLCZ1 as an immunoreactive band at approximately 77 kDa in buffalo sperm. Furthermore, immunolocalization of PLCZ1 in uncapacitated buffalo sperm showed predominant expression in the acrosome and post-acrosomal regions, with minimal reactivity in the tail region. Conversely, capacitated buffalo sperm exhibited a shift in PLCZ1 localization, with a prominent presence at the acrosomal cap region of the sperm head and reduced reactivity in the tail region. This translocation of PLCZ1 in capacitated sperm suggests early physiological activities, particularly in inducing calcium oscillation to initiate oocyte activation during fertilization. Moreover, the successful detection of PLCZ1 in buffalo sperm in the present study highlights its potential as a biological marker for screening bull fertility and genetic improvement in water buffaloes.

Keywords: Immunolocalization, oocyte activation, phospholipase C zeta 1, sperm, water buffalo.

INTRODUCTION

Fertilization is a fundamental event in all organisms, involving developmental processes such as cortical granule exocytosis, zygotic genome activation, the prevention of polyspermy, and the release of oocytes from meiotic arrest [1, 2]. These developmental events underlying oocyte activation are initiated by a series of Ca^{2+} oscillations, which are repeated increases in the intracellular concentration of free cytosolic Ca^{2+} in the egg cytoplasm [3, 4]. This pattern of Ca^{2+} oscillation is induced by a sperm-specific protein identified as Phospholipase C zeta 1 (PLCZ1) [5].

PLCZ1 was first identified in mice [6] and subsequently in other mammalian species, including monkeys, humans [7], chickens [8], pigs [9], and horses [10] and fish [11]. Over the past few decades, research efforts have refined our understanding of how PLCZ1 induces Ca^{2+} oscillations in the oocyte. PLCZ1 catalyzes the breakdown of Phosphatidylinositol 4,5 biphosphate (PIP2) to inositol 1, 4, 5-triphosphate (IP3) and diacylglycerol (DAG); IP3 subsequently binds to IP3R1 in the endoplasmic reticulum (ER) membranes, triggering Ca^{2+} release from IP3-sensitive Endoplasmic Reticulum stores. The increase in calcium ion concentration is essential for the completion of all the events of egg activation [12].

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Furthermore, several studies have investigated the association of PLCZ1 with mammalian fertility, wherein the reduction or absence of PLCZ1 within sperm has been directly linked to male infertility due to mutational inactivation or loss of PLCZ1 expression [4, 5]. In humans, higher PLCZ1 levels in sperm have been directly associated with high fertilizing ability and development, whereas low PLCZ1 concentrations are linked with infertility [4, 12]. Thus, gaining insights into the mechanism of PLCZ1 provided strong evidence that PLCZ1 is considered the primary physiological agent for fertilization and embryo development and represents a biomarker for assisted animal reproductive techniques (ART) as a prognostic and diagnostic biomarker for oocyte activation ability.

Several studies have focused on the detection and evaluation of the localization pattern of PLCZ1 to gain a comprehensive understanding of the biological activity of PLCZ1 as an oocyte activator during fertilization. In mammalian species, different localization patterns in sperm have been observed [10, 13, 14], but none have been reported in water buffalo yet. Reproduction in buffaloes is challenging; thus, understanding the role of sperm factors, particularly the PLCZ1 protein, and elucidating the mechanism by which it triggers oocyte activation and subsequent embryo development are interesting areas of progress that can impact buffalo reproduction. Thus, the present study was conducted to determine the expression of PLCZ1 in buffalo sperm and to compare its location between uncapacitated and

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capacitated buffalo sperm relative to its putative general role in oocyte activation during fertilization in various livestock species.

MATERIALS AND METHODS

Procedures for the use of animals for scientific research purposes were approved by the Ethics Committee of the Philippine Carabao Center, National Headquarters, and Gene Pool.

Detection of Buffalo PLCZ1 by Western Blot Techniques

Frozen thawed sperm (500,000) from single ejaculates from two donor bulls with two replicates per sample were used in the study.

Anti-buffalo PLCZ1 polyclonal antibody was used in western blot analysis for the detection of buffalo PLCZ1 protein. The synthetic peptides were designed based on the Buffalo PLCZ1 protein sequence: N terminal region (MAVICNQAVRITVPNPVEAC) and C terminal region (KMGESLEPASLFIYVWYIR). Frozen semen samples of water buffaloes with normal sperm parameters were used in a total protein extraction for PLCZ1 protein detection using a ReadyPrep protein extraction kit (Biorad, USA) with the addition of a protease inhibitor cocktail (Sigma-Aldrich, USA) that prevents endogenous enzymes such as proteases and phosphatases which are capable of degrading proteins in the extracts. Following extraction, the samples were subjected to sonication for 3 minutes, heated at 95°C for 5 minutes with 4x Laemmli sample buffer (Biorad, USA), and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Biorad, USA). Protein extracts equivalent to 2 x 10^6 sperm were loaded per lane into 10% Mini protean TGX stain-free gel (Biorad, USA), and transferred onto a trans-blot turbo transfer pack (Biorad, USA) using a Transblot turbo transfer system (Biorad, USA). The membrane was blocked in 6% non-fat dry milk in Phosphate Buffered Saline, 0.1% Tween 20 (PBST) and incubated overnight at 4°C with anti-PLCZ1 antibodies (1:1000). This was followed by three times washing with PBST and then incubation in secondary antibody (Goat anti-rabbit horseradish peroxidase (HRP) labeled) with 1:3000 dilutions at 4°C for 1 hour and end with thorough washing. Immunoreactivity was visualized using the Opti 4CN Colorimetric Detection kit (Biorad, USA) according to the manufacturer's directions. The membrane was visualized using a Gel Doc EZ Imager (Biorad, USA) and Image Lab V5.1.

Immunolocalization of PLCZ1 in Uncapacitated Buffalo Sperm and Induction of *In-Vitro* Capacitation and Acrosome Reaction

Frozen thawed sperm (500,000) from single ejaculates from two donor bulls, with four replicates per sample, were used in the immunolocalization studies to investigate the localization pattern of PLCZ1 in water buffalo sperm.

Uncapacitated Buffalo Sperm

A highly motile subpopulation of sperm was harvested using a swim-up procedure performed in a medium devoid of bicarbonate to minimize capacitation (non-capacitating medium (NCM): 133 mM NaCl, 5.4 mM KCl, 1 mM NaH2PO4, 0.8 mM MgSO4.7H2O, 1.8 mM CaCl2.H2O, 5.6 mM glucose, 2.5 mM sodium pyruvate, 19 mM sodium lactate and 10 mM HEPES, pH 7.4), [2].

In-Vitro Capacitation and Acrosomal Reaction

Spermatozoa were suspended in a capacitating medium containing bicarbonate and supplemented with 5mg/ml of Bovine serum albumin (BSA) (116 mM NaCl, 5.4 mM KCl, 1mM NaH2PO4, 0.8 mM MgSO4.7H2O, 1.8 M CaCl2. H2O, 5.6 mM glucose, 2.5 mM sodium pyruvate, 19 mM sodium lactate, 26 mM NaHCO3, pH 7.4 [15]. And incubated for 3h at 37℃, 5% CO^2. Capacitated sperm were then incubated in 5 µM calcium ionophore (Sigma-Aldrich, USA) for 1 hour at 37℃, 5% CO^2 to induce the acrosome reaction.

Immunofluorescence/Immunolocalization of PLCZ1

Localization of PLCZ1 in buffalo sperm by immunofluorescence was performed in ejaculated buffalo sperm using the Anti-buffalo PLCZ1 polyclonal antibody. Sperm samples were initially washed with Dulbecco's Phosphate-Buffered Saline (DPBS) by centrifugation (500 × g, 5 min). The supernatant was discarded, the semen pellet was diluted in fresh PBS, and sperms were counted using a hemocytometer. Sperm samples were fixed with 1 ml of DPBS containing 3.7% paraformaldehyde for 30 min at 4°C washed with DPBS, and permeabilized with 0.1% (v/v) Triton X-100 for 10 min at room temperature. Then, 10µL was loaded on a 0.1 % poly L-Lysine pre-coated slide (Matsunami, Japan) and allowed to settle for 20 min at 37°C. The sperms were then blocked in 5% normal goat serum (Sigma-Aldrich, USA) for 3 h at 4°C. The Anti-buffalo PLCZ1 polyclonal antibody (1:100) in 5% goat serum was added to the sperm and incubated

overnight at 4°C. After incubation, sperm samples were washed with 0.1% Tween 20 in DPBS, followed by a secondary antibody (Alexa Fluor 555 goat anti-rabbit IgG) for 1 hr at room temperature in the dark and then washed three times.

Confirmation of Sperm Nucleus and Acrosome Integrity

For the sperm nucleus staining, 5µg/ml of Hoechst 33258 was added and incubated for 10 min at room temperature and washed three times. This is followed by acrosomal sperm evaluation using fluorescein isothiocyanate- conjugated *Peanut* agglutinin (FITC-PNA, Invitrogen, Paisley, UK) for 15 min at 37°C in the dark.

The slide was mounted with Fluoroshield mounting medium (Sigma-Aldrich, USA). The slides were then analyzed for the pattern of PLCZ1 localization using an inverted fluorescence microscope (Nikon ECLIPSE Ti2, Japan), which was captured using Nikon NIS-Elements Advanced Research software.

RESULTS

Detection of PLCZ1 Protein in Water Buffalo Sperm using Western Blot

Immunoblotting was conducted to determine the presence of PLCZ1 protein in water buffalo sperm and to evaluate the specificity of the anti-buffalo PLCZ polyclonal antibody before performing immunolo-calization. The results revealed the successful detection of buffalo PLCZ1 by antibodies as an immunoreactive band of approximately 77 kDa (Figure **1**), with no other bands were detected. To determine the sensitivity and specificity of the antibody, immunoblotting using pre-immune serum was also conducted, with no protein band was detected. The result confirms the presence of PLCZ1 in buffalo sperm samples, suggesting its potential involvement in biological functions and physiological activities during fertilization by buffalo sperm.

Immunolocalization of PLCZ1 in Uncapacitated and in *In-Vitro* Capacitated-Acrosome-Reacted Buffalo Sperm

An immunofluorescence study was conducted using the same anti-buffalo PLCZ1 antibody employed in Western blot analysis. Before conducting PLCZ1 localization in both uncapacitated and capacitated-acrosome reacted sperm, the nucleus integrity was assessed to determine and select viable sperms, and

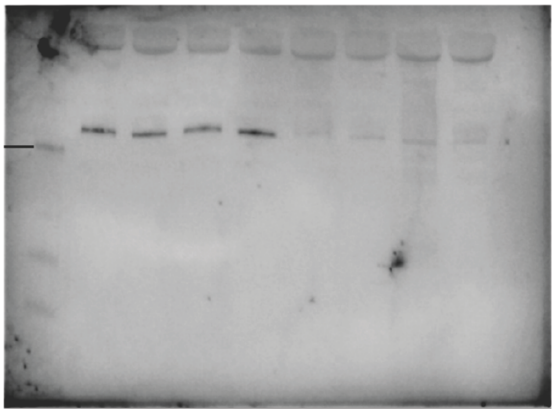

Figure 1: Buffalo PLCZ1 immunoblotting using anti-buffalo PLCZ1 antibody. An expected protein band of approximately 77 kDa was observed in a total protein extracted from water buffalo sperm. Bull 1 (lanes 1-2) and Bull 2 (lanes 3-4).

those with damaged nuclei showing a blue-white fluorescence were excluded. Likewise, the presence of acrosome was assessed, wherein the acrosome-intact spermatozoa displayed intensive green fluorescence over post-acrosomal and acrosomal regions in uncapacitated sperm, while capacitated acrosome-reacted spermatozoa showed an intensive fluorescence at the acrosomal cap (Figure **2**).

PLCZ1 in uncapacitated sperm PLCZ1 was localized at the head and tail region; however, it appeared predominantly localized at the acrosomal and post-acrosomal regions of the sperm (Figure **2**), while the PLCZ1 localization pattern remained unchanged after capacitation. On the other hand, no signal of PLCZ1 was obtained when samples were subjected to immunofluorescence in the absence of the primary antibody. Moreover, the present study investigated PLCZ1 function as an oocyte-activating factor in water buffalo, revealing compelling findings during *in-vitro* acrosomal reaction induction by treating sperm with an ionophore. Significantly, PLCZ1 demonstrated migration towards the acrosomal cap, with reduced or negligible presence observed in the post-acrosomal and tail regions. This pattern was corroborated by FITC-PNA staining post-*in-vitro* acrosome reaction, aligning with the movement of PLCZ1 protein found concentrated at the acrosomal cap in water buffalo sperm **(**Figure **2**).

DISCUSSION

The present study demonstrated the successful detection of PLCZ1 and its pattern of localization in water buffalo sperm under certain physiological conditions. The elucidation of expression and location of PLCZ1 in buffalo sperm revealed its compatibility

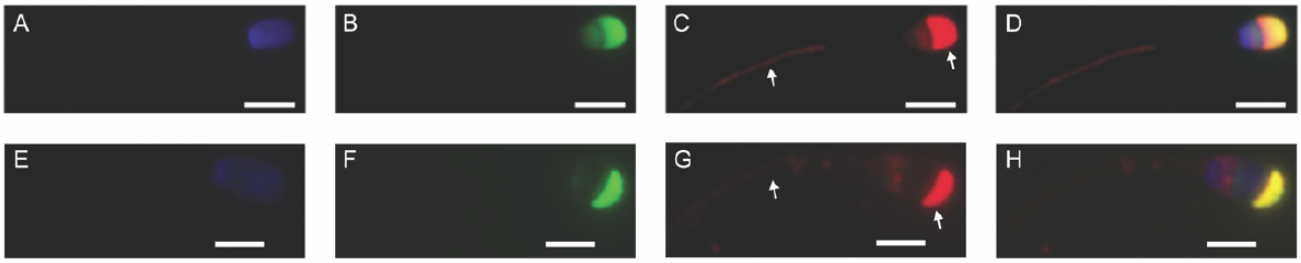

Figure 2: Representative immunofluorescence images of frozen-thawed sperm of water buffalo for PLCZ1 localization. Sperm was assessed by nucleus integrity using Hoechst 33258 (**A** & **E**), acrosome integrity using FITC-PNA (**B** & **F**), and PLCZ1 localization using Anti-buffalo PLCZ1 antibody (**C** & **G**). Uncapacitated sperm (**C**) exhibits PLCZ1 detection at the head region but with predominant localization at the acrosomal region and low reactivity at the tail region indicated by white arrows; Capacitated sperm after acrosomal reaction (**G**) exhibit movement of PLCZ1 at the post-acrosomal region indicated by white arrows. Merged images (**D** & **H**). Image magnification: 40x (**A**) and 60x (**B**). Scale bars = 5μm.

with its putative role as an oocyte activation factor. The current findings provide support to its physiological relevance as a mediator of this key process during fertilization.

The detection of full-length buffalo PLCZ1 protein revealed an immunoreactive band of approximately 77 kDa, which nearly corresponds to the range of 70-75 kDa molecular weight reported for other species studied so far, indicating a conserved structure, size, and function of PLCZ1 among species [7, 9, 16-20]. The identification of PLCZ1 in buffalo sperm in the present study provides baseline information supporting its role as a sperm-specific protein and as an oocyte activator during fertilization.

In humans, infertile individuals analyzed by the presence of PLCZ1 through western blot, whose sperm cannot induce Ca^{2+} oscillations in mouse eggs, revealed a reduced or total absence of PLCZ1 protein compared to fertile men [4, 21]. Several cases of infertility or subfertility, including oocyte activation deficiency and total fertilization failure, have been associated with the reduction or absence of PLCZ1 in other species [4, 21, 22].

Meanwhile, PLCZ1 localization in sperm has been studied in various species to determine its function and association during fertilization. The present study demonstrated that PLCZ1 is predominantly located in the acrosomal and post-acrosomal regions in buffalo sperm, which conforms to reports in human, mouse, hamster, porcine, and equine sperm [3, 10, 13, 23- 25]. The present findings, however, differ from those of human and cattle sperm, in which PLCZ1 was found to be mainly localized in the equatorial and post-acrosomal regions of the sperm [4, 21, 26, 27]. Moreover, buffalo PLCZ1 was also observed in the tail region of water buffalo sperm; however, the intensity in this region is quite low, which conforms with that observed in porcine [14] and humans [5] sperm tails. Different patterns of PLCZ1 localization in mammalian sperms might be due to their species-specific differences in terms of mechanisms of oocyte activation at fertilization [28]. In addition, previous works have indicated that the distinct regions where PLCZ1 is localized within the sperm head suggest differential functional roles for each population [17, 27, 29].

The present study is an attempt to relate the biological function of PLCZ1 as an oocyte-activating factor in water buffalo during *in-vitro* capacitation and acrosome reaction. It is interesting to note the migration of PLCZ1 from the acrosomal region to the post-acrosomal cap of buffalo sperm implying its biological positioning for impending fertilization. The present result agrees with the previous work [23], suggesting that PLCZ1 is optimally located within the part of the sperm head, which is the one that initiates the fusion of sperm with the oocyte and immediately induces the oocyte activation. Furthermore, under capacitating conditions, sperm undergo multiple biochemical events, enabling them to undergo the acrosome reaction, penetrate the zona pellucida, and reach the oocyte membrane. Consistent with studies in mice, humans, and equines, PLCZ1 demonstrates dynamic localization changes during these events, primarily being detected in the equatorial or post-acrosomal region during sperm acrosomal reaction [2, 3, 10, 30]. Therefore, it strongly suggested that this site most likely hosts the mammalian oocyte activation factor released upon fertilization [31, 32].

Meanwhile, detecting and determining the localization pattern of PLCZ1 in sperm is crucial for comprehending its biological role and assessing male fertility. In humans, PLCZ1 content in an infertile patient's sperm was undetected, failing sperm to induce calcium oscillation after being subjected to ICSI, which

can lead to failure in egg activation and sterility [17]. Moreover, abnormal morphology and a lack of acrosome in wobbler mice sperm were observed, with no prominent presence of PLCZ1 [17]. Absence or abnormal PLCZ1 structure, expression, and localization pattern underlie functional defects in sperm, especially on oocyte activation that might lead to male infertility [4, 33].

Future studies should focus on comparing the pattern of localization and immunoreactivity of PLCZ1 in water buffalo sperm with consistently high conception rates and low conception rates. In addition, quantifying immunofluorescence intensity and correlating it with bull fertility could lead to the development of a useful molecular diagnostic tool for assessing bull fertility, which can enhance selection for breeding programs in water buffaloes.

CONCLUSION

The present study demonstrated for the first time the detection and localization of PLCZ1 in water buffalo sperm under normal uncapacitated conditions and its translocation following *in-vitro* capacitation and acrosome reaction. The findings of the present study provided interesting insights into the biological mechanisms of activation and capacitation of buffalo sperm, which further emphasized its crucial importance in mammalian oocyte activation during fertilization. The new and basic information generated from the present work sparks interest in further work on the specific function of PLCZ1 in inducing an increase in calcium concentration, initiating oocyte activation, and its influence on subsequent embryonic development. The present work bears far-reaching implications for its potential in the development of diagnostic and prognostic tools for male fertility and treatment. Most importantly, bull fertility screening and selection based on PLCZ1 concentration can be a valuable technological innovation to enhance breeding and genetic improvement not only in buffaloes but in other livestock species.

AVAILABILITY OF DATA AND MATERIALS

Data and materials used for this research are available from the main author upon request.

DECLARATION OF INTEREST

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

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AUTHORS CONTRIBUTION

Roseline D. Tadeo has conceptualized and conducted the experiments, analyzed the results/data, and written the manuscript. Eufrocina P. Atabay and Edwin C. Atabay contributed to the conceptualization of the research study, as well as its finalization, review, revision, and proofreading for publication.

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