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PKG pathway is involved in the Spermine-NONOate induced acrosome reaction in buffalo spermatozoa

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Abstract
Acrosome reaction is very important reaction which occurs in capacitated spermatozoa and involves the fusion of outer acrosomal membrane with overlying plasma membrane and release of mainly acid hydrolases and renders the potential of fertilization in spermatozoa. Spermine-NONOate a nitric oxide donor also causes the AR through the synthesis of cGMP and activation of protein kinase G (PKG). In this study there is protein tyrosine phosphorylation of p20.p32, p45, p49, p69, p80 and p105 proteins. Spermine-NONOate in presence of PKG inhibitor resulted in significant inhinbitoon of AR as compared to spermine-NONOate alone (<0.05). Spermine-NONOate exhibited increased percentage of AR as compared to the control (<0.05). Therefore to spermine-NONOate caused the percent AR through activation of cGMP/PKG pathway.

Keywords: Nitric oxide, acrosome reaction, phosphorylation, PKG, buffalo

Introduction
Capacitation is the process in which the plasma membrane loses its cholesterol content and undergoes glycoprotein redistribution leading to loss of phospholipid asymmetry and increase in phospholipase activity (Boerke *et al*., 2008) [2]. Intracellular ROS including nitric oxide directly stimulates the adenylyl cyclase provoking downstream increase of protein tyrosine phosphorylation (Roy *et al*., 2008) [15]. The acrosome which appears like lysosome is a membrane bound structure derived from the Golgi apparatus located most anteriorly in the head of the sperm that forms a cap in the proximal segment of the sperm head. The outer acrosomal membrane is located subjacent to the plasma membrane and the inner acrosomal membrane is located anterior to the nuclear membrane. During acrosomal exocytosis, the outer acrosomal membrane fuses with the overlying plasma membrane and acrosomal contents are released by calcium-mediated exocytosis in response to specific signals which consist of acid hydrolases. Multivalent interactions between sperm proteins and ZP3 probably trigger this Ca^{2+}-dependent reaction. ZP3 stimulation results in opening of the sperm's T-type channels and a sustained release of Ca^{2+} from internal stores. ZP3-stimulated sperm exhibit a transiently elevated pH that may activate Ca^{2+}/calmodulin-dependent adenyl cyclases, protein phosphatases and kinases, tyrosine kinases, and phospholipases.
NO was reported to be involved in AR through the synthesis of the second messenger cGMP and the activation of kinases (PKC and protein kinase G — PKG) (Revelli *et al*., 2001) [14]. One of the molecular targets of cGMP is PKG; two different cGMP-dependent protein kinases (PKGI and PKGII) have been identified in mammals (Lohman *et al*., 1997; Pfeifer *et al*., 1999) [8, 13]. In our experiments, the PKG inhibitors, Rp-8-Br-cGMPS and Rp-8-pCPT-c-GMPS, blocked the SNP-induced AR, suggesting that the NO/cGMP pathway, which is activated by SNP stimulation, needs the activation of a PKG in order to trigger AR in human sperm (Revelli *et al*., 2001 [14]; Sharron *et al*., 2010 [16].

Materials and Methods
Semen collection and sperm culture medium
The semen were collected from six Murrah buffalo (*Bubalus bubalis*) bulls (three ejaculates from each bull) at Artificial Breeding and Research Complex, National Dairy Research Institute, Karnal, India, kept under uniform nutritional and management conditions. Semen ejaculates of +3 score or more were collected two times in a week from three buffalo bulls (three ejaculates from each bull) using artificial vagina (IMV, France) maintained at 40 °C.

Immediately after collection, mass motility were assessed by light microscopy (Eclipse-200, Nikon, Japan). Ejaculates containing spermatozoa with >80% forward progressive motility and 1 × 10^9 cells/mL were used in this study and were immediately brought to the lab and analyzed. A modified Tyrode's bicarbonate-buffered medium designated as sp-TALP [composed of 100mM NaCl, 10mM HEPES, 3.1mM KCl, 0.4mM EDTA, 0.4mM $MgCl_2 \cdot 6H2O$, 0.3mM $NaH_2PO_4 \cdot 2H_2O$, 21.6mM Na lactate, 2mM $CaCl_2 \cdot 2H2O$, 1mM Na pyruvate, 25mM $NaHCO_3$, BSA (1mg/mL for washing, 6mg/mL for culturing), pH 7.4, osmolarity: 265–270 mOsmol/kg] described by Parrish *et al.* (1988) [12] and as modified by Galantino-Homer *et al.* (1997) [3] was used in the experiments.

Processing of spermatozoa

Five hundred micro litre of freshly ejaculated semen were taken in 15 mL polypropylene tubes and washed in sp-TALP by centrifugation at 275 × g for 6 min. The seminal plasma was discarded and pellet was subjected to two washes by resuspension with 3 ml of sp-TALP (1mg BSA/mL) and centrifugation at 275× g for 5 min. The loose sperm pellet was given final wash in sp-TALP (6mg BSA/mL). The pellet was resuspended with sp-TALP (6mg BSA/mL) and the sperm concentration was determined by haemocytometer and adjusted to 100 × 10^6 cells/ml.

Sperm culture

Capacitation of buffalo spermatozoa was performed by using the protocol of Roy and Atreja (2008) [15]. Capacitation was done in a set of 1.5 mL eppendorf tube maintained at 37 °C. 250 µL of sperm suspension was added in 250 µL of sp-TALP. Twenty five million sperm cells (in a final concentration of 50× 10^6 cells) in sp-TALP (6mg BSA/mL) were used for capacitation. Sperm capacitation was induced by heparin in a concentration of 10µg/mL. All the tubes with caps open were incubated for 6h at 38.5 °C with 5% CO_2 and 85% relative humidity in air. At regular intervals, the tubes were gently mixed in order to maintain uniformity of suspension. All the suspensions were regularly checked for motility at hourly intervals by taking a 10 µL drop on pre-warmed glass slide duly covered with a cover slip and observed under an inverted bright field microscope at 200X magnification. After 6h of incubation, sperm samples were processed for the assessment of acrosome reaction.

Protein tyrosine phosphorylation in presence of PKG inhibitor

Processed and diluted spermatozoa were incubated for 6 h in Sp-TALP medium in presence of heparin (10µg/mL). At the end of incubation extent of AR was evaluated by treating the heparin capacitated spermatozoa in absence or presence of Spermine-NONOate (100 µM), PKG inhibitor (Rp-8-Br-cGMPs, 15 µM) and Spermine-NONOate+PKG inhibitor following incubation in CO_2 incubator for 15 min. Moreover, immunoblotting was performed according to the protocol given below.

SDS-PAGE and immunoblotting

Proteins from the spermatozoa were extracted according to the method of Galantino-Homer *et al.* (1997) [3] with minor modification for the buffalo spermatozoa. Protein was estimated by modified micro Lowry method. SDS-PAGE was carried out according to the method of Laemmli (1970) [6]. Eelectrophoresis was run at constant voltage of 40V until the tracking dye reaches the bottom of the gel. Two gels were run simultaneously for one type of study. After electrophoresis, one gel was subjected to staining with Coomassie Brilliant Blue R-250 and the other gel was processed for immunoblotting as described below.

The Nitric oxide induced tyrosine phosphorylated proteins in capacitated buffalo spermatozoa was detected by employing an indirect immunoblotting technique. The separated proteins on gel was transferred to Immobilon-P PVDF membrane (0.45µ) adopting the two-step transfer method of Otter *et al.* (1987) [11] duly modified by us. Next day, the membrane with the blocking solution was removed and incubated with monoclonal antiphosphotyrosine antibody [Sigma Clone PT-154, mouse IgG1, ascites fluid; diluted (1:2000) in TBS-TV] for 2h at RT with intermittent gentle agitation. The membrane was removed from first antibody solution and washed briefly (30s × 2) and then thoroughly (15 min ×4) with TBS-T with gentle agitation. The membrane was removed from the last wash solution and incubated with goat anti-mouse IgG peroxidase conjugate [Sigma; A2554 diluted (1:70,000) in TBS-TV]] for 1h at RT with intermittent gentle agitation. The membrane was removed from the second antibody solution and washed briefly (30s × 2) and then extensively (15 min × 8) with TBS-T. The immunoblot was Visualized by using Enhanced chemiluminescent reagent (Immobilon™ western chemiluminescent HRP substrate) and exposed with X-ray film. X-ray film and CBB R-250 Stained Membrane were photographed by placing the X-ray film and membrane on a white sheet of Alpha-Imager (Alpha-Innotech, USA) and captured the image. The image was then analyzed for measuring the band intensities using Alpha Ease software, version FC 6.0.1. The lanes and bands were selected manually.

Assessment of acrosome reaction

Heparin capacitated spermatozoa were incubated in the presence and absence of LPC (positive control, 100 µg/mL) or different inducers or inhibitors of the nitric oxide or protein kinase inhibitors in CO_2 incubator for 15min at 38.5ºC with 5% CO_2 in air for the induction of acrosome reaction. The samples were then subjected to dual staining procedure as described by Suraj and Atreja (2002) [19] to differentiate the physiological as well as degenerative acrosome loss.

Acrosome reaction in presence of PKG inhibitor

Heparin capacitated spermatozoa were taken and treated with Spermine-NONOate, PKG inhibitor (Rp-8-pCPT-cGMPs), Spermine-NONOate+PKG inhibitor and LPC (positive control) for 15 min and percentage AR was evaluated by counting 200 cells after dual staining.

Statistical analysis

All the experiments were repeated at least 3 times and normally distributed data were analysed by one-way ANOVA (analysis of variance). Results are expressed as the means ± S.E.M. Statistical differences between the effects of various treatments were determined by Duncan's Multiple Range Test (DMRT) using the Statistical Product and Service Solutions, version 17.0.1 software (SPSS Inc., Chicago, IL, USA). A difference with $P< 0.05$ was considered statistically significant.

Results

Effect of PKG inhibitor on protein tyrosine phosphorylation during AR

Proteins p20, p32, p45, p49, p69, p80 and p105 were tyrosine phosphorylated and had different densities as evidenced by densitometric analysis (Figure-3 and Table-1). In the absence or presence of PKG inhibitors, spermine-NONOate, PKG

inhibitor with spermine-NONOate during AR, there was phosphorylation of a specific set of proteins in the molecular weight range of p20-p105 kDA. p20 was more phosphorylated in the spermine-NONOate treated semen sample which on addition of PKG inhibitor, it is either absent in PKG inhibitor group or significantly less phosphorylated in the spermine-NONOate+PKG inhibitor. p32 protein was more significantly(*P<0.05*) phsophorylated in the spermine-NONOate treated sample which is comparable to the spermine-NONOate+PKG inhibitor, but there was significant decrease *P<0.05*) in the phosphorylation of p32 in the PKG inhibitor treated sample. p45, p49, p69, p80 were significantly (*P<0.05*) more tyrosine phosphorylation in spermine-NONOate as compared to their corresponding control, PKG inhibitor and spermine-NONOate + PKG inhibitor. Similarly p80 and p105 were significantly more tyrosine phosphorylated in spermine-NONOate treated group followed by spermine-NONOate + PKGI and PKGI. From above results it is very clear that the tyrosine phosphorylation decreases significantly after the addition of PKGI in spermine-NONOate group.

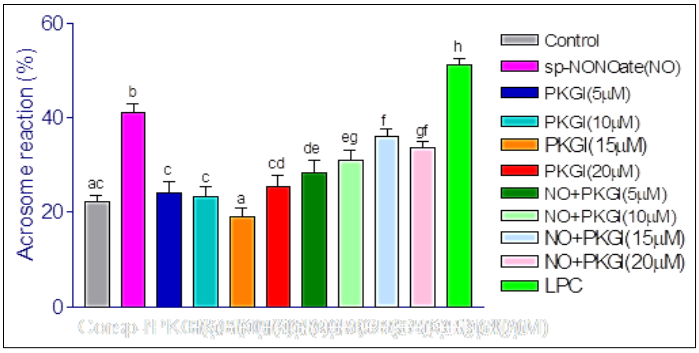

Fig 1: Effect of different concentration of PKG inhibitor on acrosome reaction. Heparin capacitated buffalo spermatozoa were incubated for 15 min in the absence and presence of different concentration of PKG inhibitor (Rp-8-pCPT-cGMPs; 5, 10, 15, 20 µM) and also with spermine-NONOate + PKG inhibitor and its effects on AR for inducing AR. LPC was taken as a positive control. Values are the mean ± SEM of three different samples. Different letters (a, b, c, d, e, f, g, h) indicate significant differences (p<0.05).

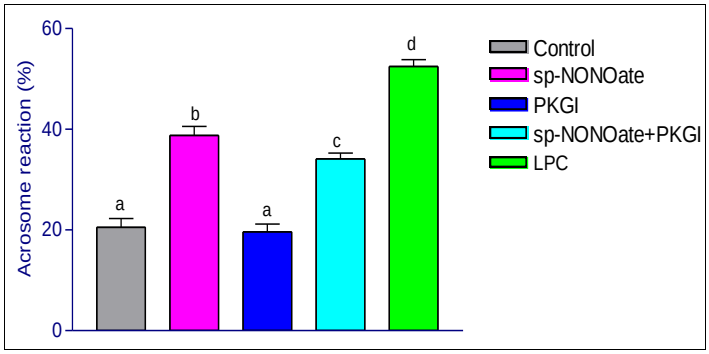

Fig 2: Effect of PKG inhibitor on acrosome reaction. Heparin capacitated buffalo spermatozoa were incubated for 15 min in the absence (control) or presence of spermine-NONOate (100 µM) or PKG inhibitor (15 µM) or combination of PKGI + spermine-NONOate or LPC (as positive control for inducing AR. Values are the mean ± SEM of three different samples. Different letters (a, b, c, d) indicate significant differences (p< 0.05).

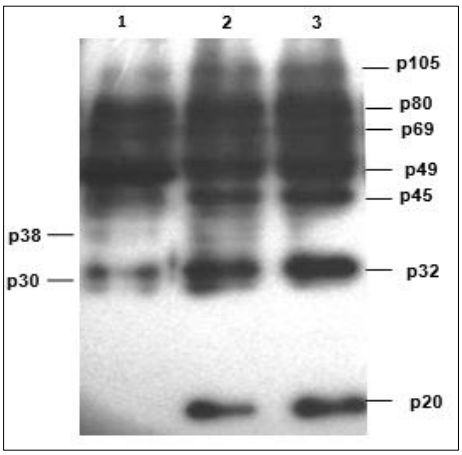

Fig 3: Effect of PKG inhibitor on protein tyrosine phosphorylation. Protein tyrosine phosphorylation during acrosome reaction in heparin capacitated buffalo spermatozoa in presence of PKG inhibitor (Rp-8-Br-cGMPs). Lane 1, 2 and 3 represents PKG inhibitor, spermine-NONOate + PKG inhibitor, spermine-NONOate, respectively. After incubation as per material and methods, sperm proteins were extracted with Laemmli sample buffer containing 5mM DTT. The extracted proteins were then resolved by 10% SDS-PAGE (10µg/lane), electro transferred to a PVDF membrane and tyrosine phosphorylated proteins were detected using an affinity purified monoclonal antiphosphotyrosine antibody. The phosphotyrosine containing proteins are marked on the right and left side of the figure (n = 3).

Table 1: Relative band intensities (Mean ± SE) of tyrosine phosphorylated proteins in buffalo spermatozoa in presence of PKG inhibitor of AR

Group	Control (LPC)	Spermine-NONOate	PKGI	PKGI + Spermine - NONOate
p105	100[a]	71.70389±2.6034[c]	58.25939±2.3333[a]	64.56709±1.7320[b]
p80	100[a]	99.54557±2.0275[b]	95.8981±1.7638[a]	98.48174±1.5275[ab]
p69	100[a]	102.6873±2.0816[c]	34.82334±1.4529[a]	82.9898±1.5275[b]
p49	100[a]	101.85127±2.9627[a]	104.4038±2.9627[a]	98.33379±2.3094[b]
p45	100[a]	98.9365±2.1858[c]	38.88889±2.0275[a]	54.60319±2.6034[b]
p32	100[a]	183.8723±1.4529[c]	132.2843±1.7320[a]	178.1082±2.6457[b]
p20	100[a]	115.5766±1.4529[b]	absent	102.6305±1.4529[a]

Values are the mean ± SEM of three different samples. Different letters ([a, b, c]) indicate significant differences ($p<0.05$).

Effect of different concentration of PKG inhibitor on acrosome reaction

The results of different concentration of PKG inhibitor (Rp-8-Br-pCPT-cGMPS; 5, 10, 15, 20 µM) are presented in Figure-1. Spermine-NONOate exhibited significantly (*P<0.05*) increased percent AR as compared to control (41.13±0.87% vs. 22.29±0.30%). Spermine-NONOate in the presence of PKG inhibitor at all above concentrations significantly (*P<0.05*) inhibited the percent AR as compared to the spermine-NONOate alone. There was maximum inhibition of percent AR in the presence of 5µM Rp-8-Br-pCPT-cGMPS as compared to the spermine-NONOate (28.52±2.43% vs. 41.13±0.87%). In the presence of PKG inhibitor alone there was slight decrease in the percent AR as compared to the control.

Effect of PKG inhibitor on acrosome reaction

Heparin capacitated buffalo spermatozoa in the absence (control) or presence of Spermine-Nonoate or PKG inhibitor or combination of spermine-NONOate + PKGI or LPC; a significant (*P<0.05*) increase was observed in the percent AR of spermatozoa with spermine-NONOate as compared to control (38.79±0.78% vs. 20.51±0.77%) and their results are shown in the Figure-2. Addition of PKG inhibitor (5 µM) to this system inhibited the percent AR significantly (*P<0.05*) than the corresponding spermine-NONOate (34.10±0.14% vs. 38.79±0.78%). Moreover in the presence of PKG inhibitor alone, the capacitated spermatozoa caused a slight decrease in the percent AR as compared to the control.

Discussion

NO acts as an intracellular signal activator of guanylate cyclase leading to physiological responses mediated by the activation of cGMP-dependent protein kinase (Murad, 1999, Siddique *et al*., 2021) [17]. Many of these responses are mediated by the activation of the protein Ser/Thr kinase, the cGMP-dependent protein kinase (PKG) (Lincoln and Corbin, 1983) [7]. The signaling pathway by which NO affects cell functions are by not only limited to the stimulation of guanylate cyclase (Hererro *et al.*, 1999) but has been shown to act via the cAMP/protein kinase (PKA) (Oddis *et al.*, 1996) [10] too. NO could cause a stimulation of PKA either through a transactivation of cGMP protein kinase or through cGMP protein kinase or through cGMP induced inhibition of cAMP degradation via cyclic nucleotide phosphodiesterase type-3 (Kurtz *et al.*, 1998) [5]. Effects of several other cGK inhibitors on PAL (PPCM induced human acrosomal loss) and SNAL (acrosomal loss induced by nitric oxide donor SNAP) were examined (Anderson *et al.*, 2009) [1]. PAL is inhibited by KT5823 (selective for cGMP dependent protein kinase-I and II) and by Rp-8-pCPT-cGMPS (inhibits cGK-I and cGK-II).

In our study AR was induced in heparin capacitated buffalo spermatozoa in absence or presence of spermine-NONOate, PKG inhibitor (PKGI), spermine-NONOate+PKGI. p20 protein was more phosphorylated in the spermine-NONOate treated semen as compared to spermine-NONOate+PKGI treated sample, but was absent in PKG inhibitor treated group. It indicates that p20 protein is dependent on the PKG protein and involves the PKG pathway.

p45, p49, p69, p80 were significantly more tyrosine phosphorylated in spermine-NONOate treated group as compared to their corresponding PKG inhibitor and spermine-NONOate+PKG inhibitor. Spermine-NONOate treated group demonstrated significantly more tyrosine-phosphorylation as compared to spermine-NONOate+PKGI (*P<0.05*). Siddique *et al*., 2012 [18] also reported the effect of Spermine-NONOate on acrosome reaction and associated protein tyrosine phosphorylation in Murrah buffalo (*Bubalus bubalis*) spermatozoa and was found to be associated with PKA mediated protein tyrosine phosphoryaltion.

In our study heparin capacitated buffalo spermatozoa were incubated in the presence of spermine-NONOate or different concentration of PKG inhibitor (Figure-1). There was significant increase in percent AR in presence of spermine-NONOate while inhibition occurs after addition of PKG inhibitor. And maximum inhibition was obtained at 5µM concentration. Our results are in conformity with the study of Revelli *et al.* (2001) [14] in which inhibitor of PKG reduced the AR in human spermatozoa. NO acts through an intracellular signal activating guanylyl cyclase and thus cGMP (Revelli *et al.*, 2001) [14], leading to physiological responses mediated by the activation of cGMP-dependent protein kinase (PKG). Among the molecular targets of cGMP are cGMP-dependent proteins kinases, PKGI and PKGII in mammals (Lohman *et al.*, 1997; Pfeiffer *et al.*, 1999) [8]. The PKG inhibitors 8-Bromoguanosine 3', 5-monophosphorothioate Rp-isomer (Rp-8-Br-cGMPS) and 8-(4-chlorophenylthio) guanosine-3', 5'-monophosphorothioate Rp-isomer (Rp-8-pCPT-cGMPS) are able to block the sodium nitroprusside-induced AR in human sperm, suggesting that the role of NO/ cGMP pathway, which is also activated by sodium nitroprusside.

Conclusion

PKG inhibitor (Rp-8-pCPT-cGMPS, 5 µM) significantly decreased (*P<0.05*) the percent acrosome reaction induced by spermine-NONOate (34.10±0.14% vs. 43.86±0.42%). In presence of Rp-8-pCPT-cGMPS (PKG inhibitor) along with spermine-NONOate, protein tyrosine phosphorylation of p20 was absent while protein tyrosine phosphorylation of p32, p45, p49, p69, p80 and p105 proteins were decreased in the presence of PKG inhibitor. Spermine-NONOate has been identified as an inducer of acrosome reaction in buffalo spermatozoa and causes the tyrosine phosphorylation of different subset of proteins in acrosome reaction. The involvement of cGMP/PKG pathway during nitric oxide

induced AR is proved by using the inhibitors and inducers of the respective pathway

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Author contributions
All the authors have equally contributed towards planning, execution of experiment and manuscript preparation.

Conflicts of interest
The authors declare that they have no conflict of interest.

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