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Physiological Concentration of Spermine-NONOate Induces Acrosome Reaction in *Bubalus bubalis* Spermatozoa

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Abstract

We have studied the effect of spermine-NONOate on acrososme reaction (AR) of buffalo spermatozoa. Heparin capacitated spermatozoa were incubated in presence of 100 μg/mL lysophosphatidyl choline (LPC, T1) or 100 μM spermine-NONOate (T2) or 1 μM 8-Br-cGMP (T3) or 15 μM ODQ (T4) or 100 μM spermine-NONOate+15μM ODQ (T5) in combination to induce AR and assessed by dual staining. AR associated protein tyrosine phosphorylation was detected by immunoblotting and AR was assessed by dual staining. Significant numbers of spermatozoa were acrosome reacted in spermine-NONOate (T2) treated cells as compared to the control ($P<0.05$). It was also observed that 1.0 μM 8-Br- cGMP caused significantly ($P<0.05$) higher percentage of AR (32.64 ± 0.37 %) as compared to other 0.1, 0.5, 2.0 μM 8-Br-cGMP. The inhibitor of sGC (ODQ) along with spermine-NONOate (T5) resulted in significantly ($p<0.05$) higher percentage of AR cells as compared to the ODQ only. Spermine-NONOate caused tyrosine phosphorylation of p20, p38, p45, p69, p80 and p105 proteins. Therefore, this study concluded that Spermine-NONOate is involved in AR of buffalo spermatozoa by phosphorylating the tyrosine proteins though activation of cGMP.

Keywords: Acrosome Reaction, Buffalo Spermatozoa, cGMP, PKG, Phosphorylation, Spermine-NONOate, Signaling

Introduction

Mammalian spermatozoa must undergo capacitation (de Lamirande *et al.*, 1997) either in female reproductive tract or in a defined media as a prerequisite to be capable of fertilizing an oocyte which renders possible interaction between spermatozoa and the zona pellucida surrounding the oocyte and the induction of acrosome reaction (AR). It is an exocytotic process involves fusion between the outer acrosomal membrane and the overlying sperm plasma membrane. It renders the sperm cells to penetrate the zona pellucida and fuse with the egg plasma membrane overlying the post acrosomal region (Yanagimachi, 1994) by involving calcium influx, actin polymerization, rise in intracellular pH, protein activation viz. phospholipases, kinases, G proteins, etc. Nitric oxide acts as protective agent (Cheruvara, *et al.*, 2019) and maintains post thaw motility and viability of spermatozoa (Siddique *et al.*, 2019).

There are several extracellular AR effector ligands viz. prostaglandins (Joyce *et al.*, 1987), lysophosphatidylcholine (LPC; Fleming and Yanagimachi, 1981), zona pellucida (Yanagimachi, 1994), odoxaquinoline (ODQ, sGC inhibitor; Revelli *et al.,* 2001), 8-Br-cGMP (cGMP analogue; Santos-sacchi and Gordon, 1980) and H-89 (an inhibitor of PKA) or db-cAMP, an analogue of cAMP (Hyne and Garbers, 1979; Mrsny and Meizel, 1980; Fraser, 1981; Jonge *et al.,* 1991; Bielfeld *et al.,* 1994). Exogenous Nitric oxide (NO•) also induce hyperactivation (Herrero and Gagnon, 2001; Yeoman *et al*., 1998), capacitation in buffalo (Roy and Atreja, 2008) and acrosome reaction in human (Herrero *et al.,* 1999), rabbit (Guzman-Grenfell *et al.*, 1999), mouse (Herrero *et al.*,1997) and bovine spermatozoa (Zamir *et al.*, 1995; Rodriguez *et al.*, 2005). During AR, activation of kinases like Protein kinase-A (PKA), Protein kinase-C (PKC), Protein kinase-G (PKG) and Tyrosine kinases (PTK) is downstream to the production and/or activation of early second messengers (Liguori *et al*., 2005; Revelli *et al*., 2001). Nitric oxide is a free radical synthesized from the enzymatic conversion of L-arginine to L-citrulline by NADPH dependent Nitric oxide synthases (NOSs). Due to its low molecular weight and lipophilic nature, diffuses quickly through lipid membranes and is implicated in a variety of physiological cellular signaling mechanisms in many tissues by activating guanylyl cyclases. There are reports that reveal that a cGMP pathway is involved in the mammalian sperm acrosome reaction (Revelli *et al*., 2001, Rodriguez *et al*., 2005). Actions of PKG in spermatozoa have been also shown to activate protein tyrosine kinases (Willipinski-Stapelfeldt *et al*., 2004) and leads to protein tyrosine phosphorylation. Protein tyrosine phosphorylation is a post translational modification which is responsible for different sperm functions like capacitation and acrosome reaction. Tyrosine phosphorylation and its up regulation by cAMP have been associated with capacitation, AR and motility changes of spermatozoa (Bajpai and Doncel, 2003). Nitric oxide is also involved in acrosome reaction of buffalo spermatozoa by causing the tyrosine phosphorylation of proteins mainly p17 and p20 and through activation of cAMP/PKA pathway (Siddique and Atreja, 2012).

Information about the role of NO• and involvement of cGMP/PKG pathway on sperm acrosome reaction are very limited in buffalo spermatozoa. Like in human and mouse, NO• might play a role in buffalo sperm acrosome reaction. So, the present study was conducted to determine the role of NO• signalling in buffalo sperm acrosome reaction in terms of protein tyrosine phosphorylation and its crosstalk with sGC/PKG pathway.

Materials and Methods

Semen Collection

Semen was collected twice in a week from six Murrah buffalo bulls in replicate of 4-5 years age (three ejaculates from three bull chosen at random) using artificial vagina (IMV, France) maintained at Artificial Breeding Research Centre, National Dairy Research Institute, Karnal, India, under uniform nutritional conditions. Semen ejaculates were assessed immediately for mass and individual motility and semen containing >80% forward progressive motility and 1×10^9 cells/ mL was used in this study.

Sperm Culture Medium

The sp-TALP media was prepared as described by Parrish *et al.* (1988) and modified by Galantino-Homer *et al.* (1997). This is a modified Tyrode's bicarbonate-buffered medium (pH 7.4, osmolarity: 280-285 mOsmol/kg). Stock media was devoid of Ca^{2+}, BSA, pyruvate and bicarbonate and it contains 100mM NaCl, 10 mM HEPES, 3.1 mM KCl, 0.4 mM EDTA, 0.4 mM $MgCl_2\cdot6H2O$, 0.3 mM $NaH_2PO_4\cdot2H_2O$, 21.6 mM Na lactate, 2 mM $CaCl_2\cdot2H2O$, 1 mM sodium pyruvate, 25 mM $NaHCO_3$ and BSA. Media containing 1 mg/mL BSA for washing and 6 mg/mL BSA

for culturing was used. Working sp-TALP medium was supplemented with Ca^{2+} (2 mM), pyruvate (1 mM), $NaHCO_3$ (25 mM) and sodium lactate (21.6 mM).

Semen Processing

Collected semen was transported to the laboratory within 5-10 min and split into 0.5 mL fractions, and diluted with sp-TALP in 1:6 ratios. These samples were washed with centrifuge twice with 3 mL of sp-TALP to remove seminal plasma. The pellet was suspended with sp-TALP (6 mg BSA/mL) and the sperm concentration was adjusted to 100 $\times 10^6$ cells/ml.

Sperm Capacitation and Culture

Capacitation of buffalo spermatozoa was performed by following protocol of Roy and Atreja (2008). Twenty-five million cells from sperm suspension (100$\times 10^6$ cells/mL) were added to 250 µL of sp-TALP (6 mgBSA/mL) and heparin (10 µg/mL) was added to induce capacitation. The tubes were incubated at 38.5°C with 5% CO_2 and 85% relative humidity in air for 6 h with regular and gentle mixing in order to maintain uniformity of suspension. Capacitated sperm samples were processed for the assessment of acrosome reaction after 6 h of incubation.

Assessment of Acrosome Reaction in Presence of Its Modulators

In order to induce acrosome reaction, heparin capacitated spermatozoa were treated in absence (negative control) or presence of 100g/mL Lysophosphatidyl choline (LPC, T1) or 100 µM Spermine-NONOate(Z)-1-[N-(3-Ammoniopropyl)-N-[4-(3-aminopropylammonio)butyl]amino]1- diazen-ium-1,2-diolate; T2) a modulator of nitric oxide or 1 µM 8-Br-cGMP (8- Bromoguanosine- 3', 5'- cyclic monophosphate, T3) an analogue of the cGMP or 15 µM ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, T4) a highly selective, irreversible, heme-site inhibitor of soluble guanylyl cyclase or Spermine-NONOate+ODQ (T5) in combination. Different concentrations of sGC inhibitor (5, 10, 15 and 20 µM ODQ) were applied to evaluate the optimum inhibitory concentration of ODQ on AR. Similarly, different concentration of 8-Br-cGMP (0.1, 0.5, 1.0 and 2.0 µM) were applied to evaluate the optimum modulator concentration of ODQ on AR. Lysophosphatidyl choline (LPC) an inducer of acrosome reaction in capacitated cells only was used as positive control in the concentration of 100µg/mL. The sperm cells from these groups were further incubated for 15 min at 38.5°C with 5% CO_2 and 85% relative humidity. The sperm cells were then subjected to dual staining using trypan blue and Giemsa stain as described by Sidhu *et al.* (1992). A minimum of 200 cells per slides were counted for detection of live-acrosome intact cells (Acrosome-Pink/ Post acrosome-colourless) or live-acrosome reacted (Acrosome-colourless/ Post acrosome-colourless) cells (Fig. 1).

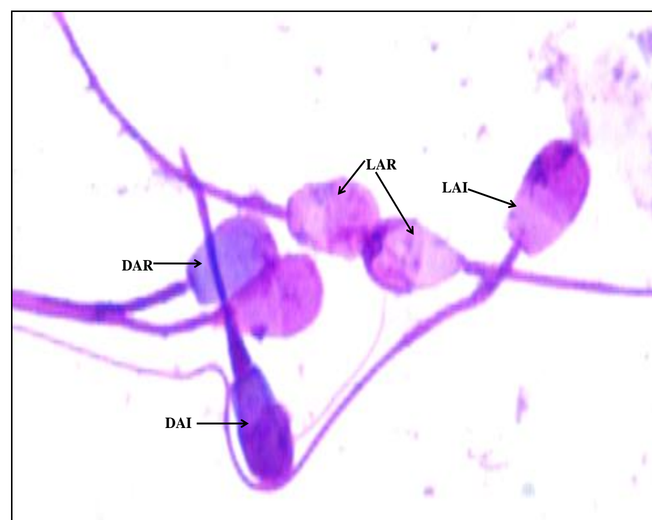

Figure 1: Dual staining (trypan blue and giemsa stain) pattern for assessing the spermine-NONOate induced acrosome reaction of buffalo spermatozoa. Four types of staining patterns were observed. i) Live spermatozoa with acrosome intact (LAI, Pink acrosome and unstained post-acrosomal region); ii) Live acrosome reacted (LAR, unstained acrosomal and post-acrosomal region); iii) Dead spermatozoa with acrosome intact (DAI, Pink acrosome and bluish-brown post-acrosomal region); iv) Dead spermatozoa with acrosome reacted or lost (DAR, unstained acrosomal region and blue post acrosomal region).

Modulators of acrosome reaction to be tested with spermatozoa were dissolved either in sp-TALP or dimethyl sulfoxide (DMSO) or alcohol depending on their solubility. The concentration of DMSO in the incubation media was kept below 1% (v/v), a condition that does not affect sperm viability, motility and the capacitation (O' Flaherty *et al.,* 2004).

Effect of Modulators of Acrosome Reaction on Protein Tyrosine-Phosphorylation

Proteins were extracted from acrosome reacted spermatozoa following the method of Galantino-Homer *et al.* (1997) and total protein concentration was estimated by method of Lowry *et al.* (1951). Proteins were separated in 10% uniform SDS-PAGE (w/v) (Laemmli, 1970) and transferred to Immobilon-P PVDF (Polyvinylidine difluoride) membrane (Otter *et al*., 1987). Equal loading and transfer efficiency of proteins was checked by membrane staining with 0.5% ponceu S staining (Salinovich and Montelaro, 1986). The membrane was blocked in 5% skimmed milk (w/v) and incubated with monoclonal antiphosphotyrosine antibody (Sigma: 1869, Clone pT-154, diluted (1:2000) in TBS-TV) for 2 h at room temperature. After washing with TBS-T, membrane was incubated with goat anti-mouse IgG-peroxidase conjugate (Sigma: A2554, dilution (1:80,000) in TBS-TV) for 1 h and washed again with TBS-T. Subsequently, the bound peroxidase activity was visualized by the Immobilon Western blotting chemiluminescent detection reagents (Millipore Corporation, Billerica, MA, USA) according to manufacturer's instructions using Kodak X-OMAT-AR X-ray films. Duplicate blots were also probed with the secondary antibody alone to rule out any non-specific binding to transferred proteins. After chemiluminescence detection, the X-ray films were photographed in a gel documentation system (AlphaInnotech® HP, Alpha Innotech Corporation, San Leandro, CA, USA) and densitometric analysis was performed with Multi Gauge analysis software (Alpha Imager, AlphaInnotech® HP, CA, USA). The density of bands measured in control sperms were set as a base value of 100 and relative density in bands of treatment groups were calculated.

Statistical Analysis

All the experiments were repeated at least 3 times and normally distributed data were analyzed by 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 Different Concentration of sGC Inhibitor and 8-Br-cGMP on Acrosome Reaction

Effect of Different Concentration of sGC Inhibitor on Acrosome Reaction

Heparin capacitated spermatozoa were incubated in the absence or presence of different concentration of sGC inhibitor (5, 10, 15, 20 µM ODQ) and also along with spermine-NONOate to evaluate the effect of sGC inhibitor on AR (Fig. 2). It was observed that ODQ at different concentrations with spermine-NONOate (spermine-NONOate+ODQ) inhibited the percent AR as compared to the spermine-NONOate alone. There was maximum inhibition of percent AR in the presence of 10 µM ODQ+spermine-NONOate as compared to the spermine-NONOate (24.87±1.77% vs. 39.62±0.12 %). In the presence of sGC inhibitor alone there was also slight change in the percent AR as compared to the control.

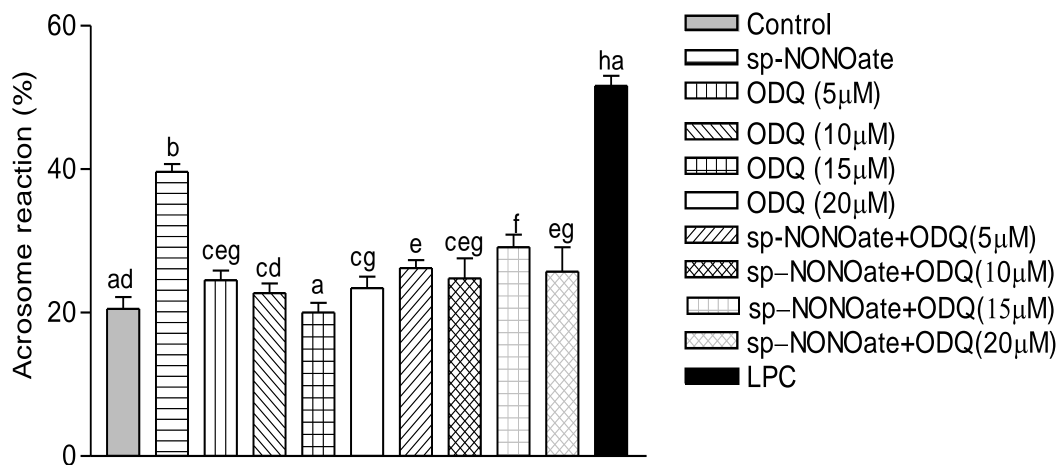

Figure 2: Effect of different concentration of sGC inhibitor (ODQ) on acrosome reaction. Heparin capacitated spermatozoa were incubated in the absence (control) or presence of spermine-NONOate, LPC and different concentration of sGC inhibitor (ODQ; 5, 10, 15, 20 µM) and along with spermine-NONOate to evaluate the effect of sGC inhibitor on AR. Values are the mean ± S.E.M. of three different samples. Different letters ([a, b, c, d, e, f, g, h]) indicate significant differences ($p<0.05$).

Effect of Different Concentration of 8-Br-cGMP on Acrosome Reaction

Effect of different concentration of 8-Br-cGMP (0.1, 0.5, 1.0 & 2.0 µM) on AR was determined and results are presented in Fig. 3. 8-Br-cGMP at 1.0 µM concentration caused significantly ($P<0.05$) higher percentage of AR (32.64±0.37 %) followed by 2.0 µM (27.25 ±0.24 %), 0.5 µM (25.34±0.38 %), and 0.1 µM (22.50±0.26%). Spermine-NONOate and LPC demonstrated significantly ($P<0.05$) higher percentage of AR as compared to the control.

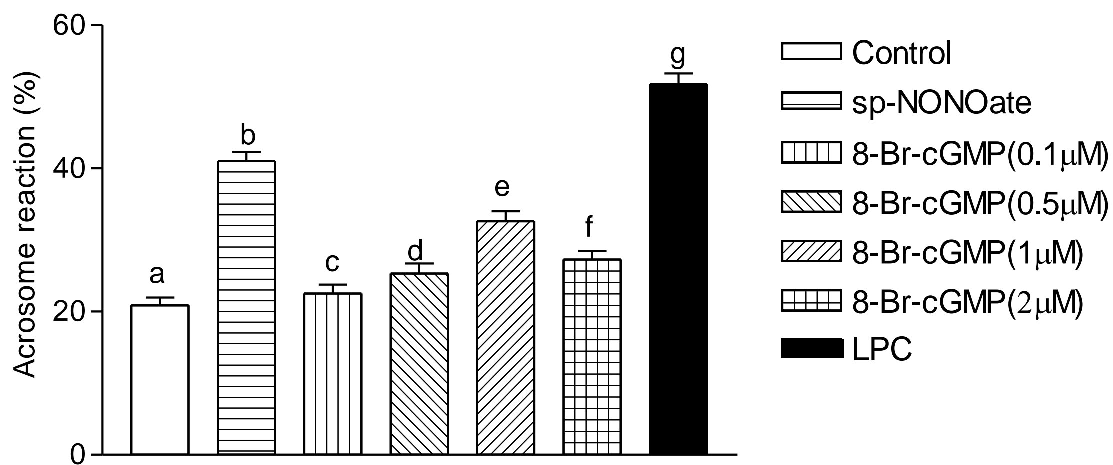

Figure 3: Effect of different concentration of 8-Br-cGMP (an analogue of cGMP) on acrosome reaction. Heparin capacitated spermatozoa were incubated for 15 min in the absence (control) or presence of spermine-NONOate, LPC (positive control) and different concentration of 8-Br-cGMP (0.1, 0.5, 1.0 and 2.0 µM) for inducing AR. Values are the mean ± S.E.M. of three different samples. Different letters ([a, b, c, d, e, f, g]) indicate significant differences ($p<0.05$).

Effect of Modulators (Spermine-NONOate, ODQ and 8-Br-cGMP) on Acrosome Reaction

Results of acrosome reaction of heparin capacitated spermatozoa in the absence (control) or presence of LPC (T1), spermine-NONOate (T2), 8-Br-cGMP (T3), ODQ (T4) and spermine-NONOate+ODQ (T5), are shown in Fig. 4. A

significant (*P<0.05*) decrease in the percentage of AR was observed in T5 as compared to the T2 (30.37±0.90% vs. 39.94±0.13 %). 8-Br-cGMP caused the increase in percent AR as compared to the control. Moreover, spermine-NONOate, 8-Br-cGMP and LPC significantly (*P<0.05*) increased the percent AR as compared to the negative control (39.44±1.13%, 31.99±0.06%, 51.76±0.39% vs. 20.69±0.66 % respectively).

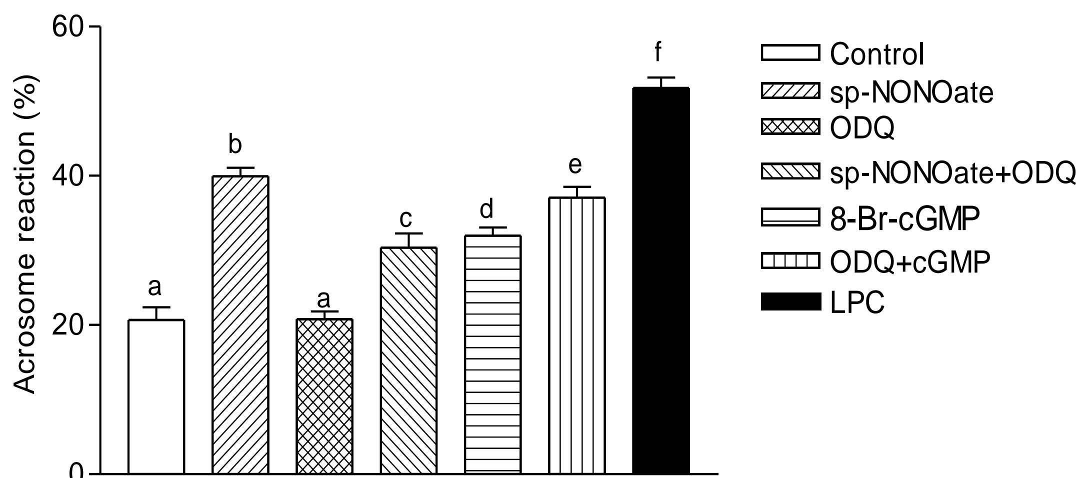

Figure 4: Effect of modulators (spermine-NONOate, ODQ, spermine-NONOate+ODQ, 8-Br-cGMP) on acrosome reaction. Heparin capacitated spermatozoa were incubated for 15 min in the absence (control) or presence of spermine-NONOate (100µM), ODQ (15 µM), spermine-NONOate+ODQ, 8-Br-cGMP (1µM), spermine-NONOate+8-Br-cGMP for inducing AR. LPC was taken as positive control. Values are the mean ± S.E.M. of three different samples. Different letters ([a, b, c, d, e, f]) indicate significant differences (*p<0.05*).

Effect of Modulators (Spermine-NONOate, ODQ and 8-Br-cGMP) on Protein Tyrosine Phosphorylation During AR

Protein tyrosine phosphorylation of spermatozoa was studied the in the absence (control) or presence of LPC (T1), spermine-NONOate (T2), 8-Br-cGMP (T3), ODQ (T4) and spermine-NONOate+ODQ (T5), as shown in Fig. 5. Proteins p20, p30, p32, p38, p45, p49, p69, p80 and p105 were tyrosine phosphorylated and had different levels of phosphorylation as evidenced by densitometric analysis (Table-1). In the absence or presence of sGC inhbitor and modulators during AR, there were tyrosine phosphorylations of specific set of proteins in the molecular weight range of p20-p105 kDa. In the presence of spermine-NONOate p20, p30, p32, p38, p45, p49, p80 and p105 were significantly (*P<0.05*) phosphorylated. However, in the presence of ODQ, there was significant (*P<0.05*) inhibition of tyrosine phosphorylation of p38, p45, p49, p69 and p80 in addition to the partial inhibition of the protein tyrosine phosphorylation of p32; which on the addition of the spermine-NONOate could reverse the inhibition of tyrosine phosphorylation caused due to ODQ. 8-Br-cGMP significantly (*P<0.05*) phosphorylated most of the proteins viz. p20, p32, p38, p45, p49, p69, p80 and p105.

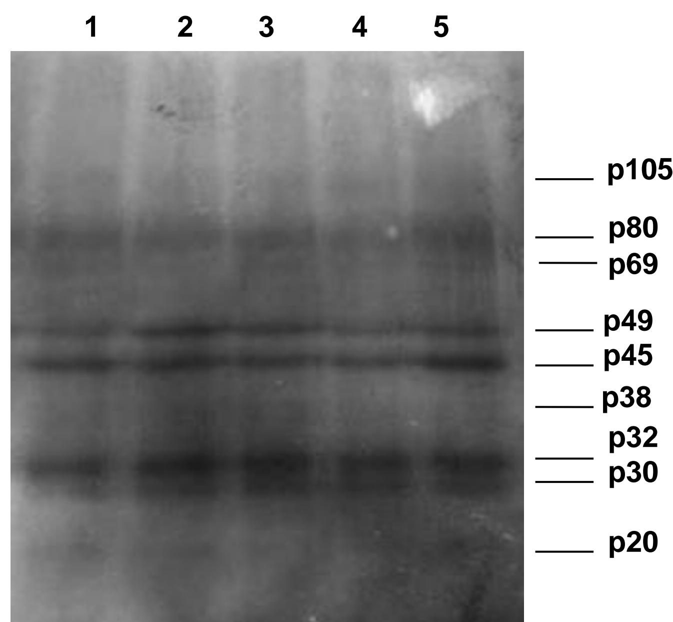

Figure 5: Effect of modulators (spermine-NONOate, ODQ, spermine-NONOate+ODQ, 8-Br-cGMP) on protein tyrosine phosphorylation. Protein tyrosine phosphorylation during acrosome reaction in heparin capacitated buffalo spermatozoa in presence of sGC inhibitor (ODQ). Lane 1, 2, 3, 4 and 5 represents LPC (control, T1), spermine-NONOate (T2), 8-Br-cGMP (T3), ODQ (T4), Spermine-NONOate+ODQ (T5), 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), electrotransferred 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 side of the figure (n=3).

Table 1: Relative Band Intensities (Mean ± SE) of Tyrosine Phosphorylated Proteins in Buffalo Spermatozoa in presence of modulators (spermine-NONOate, ODQ, spermine- NONOate+ODQ, 8-Br-cGMP) of AR

Group	Control (LPC)	Spermine-NONOate	8-Br-cGMP	ODQ	Spermine-NONOate+ODQ
p105	100	84.95334±1.492^{a}	87.13064±1.452^{a}	67.59979±1.722^{b}	86.52048±1.182^{a}
p80	100	101.287±0.881^{a}	103.219±0.880^{a}	87.33664±0.881^{b}	107.513±1.154^{d}
p69	100	108.1107±1.082^{a}	107.8389±2.432^{a}	94.6934±1.345^{b}	132.7975±1.954^{c}
p49	100	92.87403±1.154^{a}	98.50276±0.577^{b}	79.1737±0.881^{c}	87.56276±0.881^{d}
p45	100	94.36535±1.154^{a}	94.91082±1.154^{a}	84.54699±1.154^{c}	98.36361±1.452^{d}
p38	100	85.66821±1.465^{a}	86.42931±1.459^{a}	68.00826±1.522^{b}	73.91641±1.185^{c}
p32	100	89.30481±1.154^{a}	102.8503±1.420^{b}	86.07487±0.810^{c}	99.10695±0.881^{c}
p30	100	81.9369±2.027^{a}	104.5628±1.76^{b}	75.3821±1.15^{c}	80.58541±1.20^{d}
p20	100	86.717±0.88^{a}	78.42649±1.52^{b}	65.4737±0.75^{c}	68.4252±1.20^{c}

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

Discussion

In the early stages of capacitation, there are production of reactive oxygen species like NO (Herrero *et al*., 2000), which could act as an intracellular messenger by activating the soluble guanylyl cyclase (Murad, 1994), resulting in the increase in cGMP in the cytoplasm with the subsequent activation of CNG (cyclic nucleotide-gated) channels and PKG. And it has been proposed that the signalling pathway that involves CNG channels activation by cGMP should be one of the first events that occurred during capacitation, providing an important role for the cGMP pathway in the mouse sperm physiology (Cisneros-Mejorado *et al.,* 2014). Uncapacitated spermatozoa produce low levels of NO, whereas under capacitating conditions, a time-dependent increase in NO synthesis has been observed (Zhang and Zheng, 1996). *In vitro* studies have shown that low concentrations of NO enhance the AR of mouse (Herrero *et al*., 1997) and bull (Zamir *et al*., 1995) spermatozoa, and also increase the zona pellucida-binding ability of human spermatozoa (Sengoku *et al.,* 1998). Activation of endothelial NOS (Nitric oxide synthase) is implicated

in the follicular fluid induced AR of human spermatozoa (Revelli *et al*., 1999). Moreover, NO donors such as sodium nitroprusside, 3, 3-bis (aminoethyl)-1-hydroxy- 2-oxo-1-triazene (DETA-NONOate), and *S*-nitroso-*N*-acetylpenicillamine are able to stimulate the AR of human spermatozoa in a specific and dose-dependent manner (Revelli *et al*., 1999; Joo *et al.*, 1999).

In our study 100 µM Spermine-NONOate also induced significantly ($P<0.05$) higher percentage of AR. Addition of ODQ to spermine-NONOate (T5) caused decrease in AR at 10 µM concentration. And addition of 8-Br-cGMP significantly ($P<0.05$) increased the AR. Hence, these findings suggest that sGC inhibitor, inhibited the AR induced by spermine-NONOate and recovered by the addition of 8-Br-cGMP. Our findings are in agreement with the Revelli *et al.* (2001) that the AR-inducing effect of sodium nitroprusside is abolished by the guanylate cyclase inhibitors LY 83583 and ODQ can be recovered by the addition of 8-Bromo-cGMP (Revelli *et al.,* 2001). Hossein *et al.* (2010) investigated the effects of ODQ and ODQ+NOC-18 (3, 3-Bis (aminoethyl)-1-hydroxy-2-oxo-1 triazine; NO donor which release NO at a slower rate) on the kinematic parameters of epidymal ram and there was significant decrease in the different kinematic parameters and suggested that the physiological function of nitric oxide on motion parameters of epididymal sperm is mediated via guanylyl cyclase/ cGMP pathway in ram. In his experiment, sGC inhibitor (ODQ) blunted the GSNO (S-nitrosoglutathione)-elicited motility and abolished the increase in intracellular cGMP induced by GSNO (Miraglia *et al.,* 2011). The treatment with the cell-permeating cGMP analogue 8-B-cGMP, which augmented the nearly fourfold intracellular content of cyclic nucleotide, strongly increased the motility. Moreover, 8-Br-cGMP reversed the inhibitory effect of ODQ on the GSNO-evoked increase in motility, confirming that ODQ inhibited sperm motility by lowering the intracellular level of cGMP (Miraglia *et al.,* 2011).

ODQ (15 µM) significantly ($P<0.05$) inhibited the tyrosine phosphorylation of p20, p30, p32, p38, p45, p49, p69, p80 and p105 proteins caused by spermine-NONOate induced AR. Conversely, 8-Br-cGMP significantly increased the tyrosine phosphorylation of most of the above proteins. Our observations are in conformity with the study of Revelli *et al.* (2001) that the AR inducing effect of sodium nitroprusside is abolished by the guanylate cyclase inhibitors LY 83583 and ODQ and can be recovered by the addition of 8-Br-cGMP (Miraglia *et al.,* 2011). And there are more evidences of cGMP synthesis and its involvement during NO-induced AR in human spermatozoa (Zamir *et al*., 1995). Our study reveals that nitric oxide released by spermine-NONOate is involved in buffalo sperm acrosome reaction as suggested by different tyrosine phosphorylation pattern in presence of modulators (spermine-NONOate, ODQ and 8-Br-cGMP) of acrosome reaction. The tyrosine phosphorylation pattern suggests that p20, p30, p32, p38, p45, p49, p69, p80 and p105 proteins are cGMP/ PKG pathway dependent and mediated through generation of nitric oxide from spermine-NONOate during acrosome reaction. One of the molecular targets of cGMP is PKG; two different cGMP-dependent protein kinases (PKGI and PKGII) have been identified in mammals (Lohmann *et al.,* 1997; Pfeifer *et al.,* 1999). In our experiments, the sGC inhibitor, ODQ, blocked the spermine-NONOate-induced AR, suggesting that the NO/cGMP pathway, which is activated by spermine-NONOate stimulation to trigger AR in buffalo spermatozoa.

Conclusion

This study concluded that Spermine-NONoate is involved in AR of buffalo spermatozoa by phosphorylating the tyrosine proteins though activation of cGMP.

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Conflict of Interests

There is no conflict of interest.

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