https://doi.org/10.56825/bufbu.2025.4414469

GENISTEIN DECREASES THE NITRIC OXIDE INDUCED ACROSOME REACTION BY INHIBITING TYROSINE PHOSPHORYLATION IN MURRAH BUFFALO SPERMATOZOA

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Received: 29 June 2021
Accepted: 24 December 2024

ABSTRACT

Spermine-NONOate, a nitric oxide donor, contributes to various physiological functions, including the acrosome reaction (AR) at physiological levels. It triggers AR by enhancing tyrosine phosphorylation in proteins ranging from 20 to 105 kDa. Genistein, an isoflavonoid known to inhibit protein tyrosine kinase, significantly ($P<0.05$) reduces the AR percentage compared to Spermine-NONOate. Furthermore, LPC alone markedly increases the AR percentage ($P<0.05$) relative to the control (51.36±1.03% vs. 19.09±1.38%). Spermine-NONOate treatment elevates phosphorylation in proteins p20, p30, p38, p80, and p105, but this phosphorylation is significantly decreased ($P<0.05$) when genistein is present. Notably, p20 and p30 show higher phosphorylation in the Spermine-NONOate group but are absent in both the genistein-only and Spermine-NONOate+genistein groups, with p30 specifically undetectable after genistein treatment. In contrast, proteins p80 and p105 experience substantial tyrosine phosphorylation in the Spermine-NONOate group, which diminishes significantly ($P<0.05$) with genistein. This decrease in tyrosine phosphorylation during AR in the presence of genistein suggests its inhibitory effect on nitric oxide-induced AR, indicating that nitric oxide facilitates AR in buffalo spermatozoa through protein tyrosine kinase-dependent phosphorylation.

Keywords: *Bubalus bubalis*, buffaloes, Spermine-NONOate, acrosome reaction, protein Tyrosine phosphorylation, genistein

INTRODUCTION

Nitric oxide (NO) donors are recognized for inducing the acrosome reaction in sperm from

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both humans (Herrero *et al*., 1999) and rabbits (Guzman-Grenfell *et al*., 1999). Nitric oxide synthase (NOS) has been identified in the acrosomes and tails of mouse sperm (Herrero *et al*., 1997) and in human sperm (Lewis *et al*., 1996). High doses (0.01 to 1.0 mM) of sodium nitroprusside, a NO donor, can impair motility and viability in human spermatozoa, whereas lower doses (10 to 100 nM) support capacitation without influencing motility (Sengoku *et al*., 1998; Siddique and Atreja, 2013; Siddique *et al*., 2019). Joo *et al*. (1999) reported that sodium nitroprusside reduces sperm motility and hyperactivation at concentrations of 0.1 to 1.0 mM, while promoting acrosome reaction at levels of 0.01 to 1.0 mM (Revelli *et al*., 1999). Furthermore, spermine-NONOate, another nitric oxide donor, stimulates the PKA/PKG pathway, substantially enhancing the acrosome reaction (Siddique and Atreja, 2012; Siddique *et al*., 2021).

Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), an isoflavone derived from soy products and other legumes, is commonly consumed in Asian diets (Ronis, 2016). This natural compound possesses a variety of beneficial properties, including antioxidant, anti-inflammatory, antiangiogenic, proapoptotic, and antiproliferative effects, which support its potential use in cancer prevention and treatment (Kim *et al*., 2014; Ganai and Farooqi, 2015). Extensive research has focused on understanding the molecular mechanisms of genistein. It targets key proteins such as caspases, Bcl-2, Bax, and NF-κB, as well as inhibitors of NF-κB. Genistein also affects important signaling pathways, including PI3K/Akt, ERK 1/2, MAPK, and Wnt/β-catenin (Tuli *et al*., 2019).

High levels of phytoestrogens can impact male fertility (Dixon, 2004). These compounds, which are present in plants like soybeans, fava beans, lupines, and clover, bind to estrogen receptors. Genistein, an isoflavonoid with estrogen-like effects, inhibits protein tyrosine kinases (PTK), enzymes that phosphorylate tyrosine residues on membrane-bound receptors involved in signal transduction (Dixon, 2004; Akiyama *et al*., 1987). Tyrosine phosphorylation is crucial for sperm functions such as capacitation (Visconti *et al*., 1998). In contrast, epididymal proteins that cause decapacitation reduce protein tyrosine phosphorylation (Roberts *et al*., 2003), and seminal plasma decreases the percentage of sperm exhibiting hyperactivated motility by lowering tyrosine phosphorylation (Leyton *et al*., 1992). PTK inhibitors can also interfere with exocytosis and sperm penetration of the zona pellucida (Leyton *et al*., 1992; Kirkman-Brown *et al*., 2002; Pukazhenthi *et al*., 1998).

High concentrations of genistein and beta-lapachone have been shown to suppress the acrosome reaction in rats by causing cytotoxic damage to the sperm cell membrane (Agarwal *et al*., 2004). Furthermore, genistein exposure has been significantly associated with idiopathic male infertility (Queiroz and Waissmann, 2006). Increased genistein levels have been observed to decrease the motility of spermatozoa in mice, with similar effects reported in human sperm (Bajpai and Doncel, 2003). Genistein interferes with tyrosine kinase activity by competing with ATP for the enzyme's active site and also inhibits the acrosome reaction induced by sodium nitroprusside. This suggests that protein tyrosine kinase is involved in the acrosome reaction and the exocytotic processes triggered by nitric oxide in capacitated sperm cells (Leclerc *et al*., 1997). The reduction in acrosome reaction observed with a specific PTK inhibitor further reinforces the role of protein tyrosine kinase in capacitation and the acrosome reaction

in bovine sperm (Rodriguez *et al*., 2005). This led us to investigate the impact of genistein on the acrosome reaction in buffalo spermatozoa.

MATERIALS AND METHODS

Semen collection and sperm culture

Semen was collected twice a week from six Murrah buffalo bulls (*Bubalus bubalis*) at the Research and Artificial Breeding Centre of the National Dairy Research Institute in Karnal, India, with each bull providing three ejaculations. Only ejaculates meeting specific criteria of sperm score of +3 or higher, progressive forward motility exceeding 80%, and a concentration of 1×10^9 cells/mL were used in the study. The experiments employed a modified Tyrode Bicarbonate Buffer Media, spTALP, which consists of 100 mM NaCl, 10 mM HEPES, 3.1 mM KCl, 0.4 mM EDTA, 0.4 mM MgCl2 6H2O, 0.3 mM NaH2PO4 2H2O, 21.6 mM Na lactate, 2 mM CaCl2, pyruvate, 25 mM NaHCO3, and BSA (1 mg/mL for washing, 6 mg/mL for culture). This medium has a pH of 7.4 and an osmolality of 265 to 270 mOsmol/kg, as described by Parrish *et al*. (1988) and reviewed by Galantino-Homer *et al*. (1997). Prior to use, the medium was equilibrated in a CO_2 incubator (Shel Lab: 24242, water jacket, Sheldon Manufacturing Inc., USA) for one hour.

Processing and capacitation of spermatozoa

Freshly collected semen (500 µL) was placed in a 15 mL polypropylene tube and washed with sp-TALP by centrifugation at 275 × g for 6 minutes. After removing the seminal plasma, the pellet was washed twice with 3 mL of sp-TALP containing 1 mg BSA/mL, with each wash followed by centrifugation at 275 × g for 5 minutes. The sperm were then washed once more with sp-TALP containing 6 mg BSA/mL. Following the final wash, the pellet was resuspended in sp-TALP (6 mg BSA/mL), and sperm concentration was determined using a hemocytometer and adjusted to 100×10^6 cells/mL. The semen collection procedure followed Roy and Atreja (2008). To induce capacitation, 10 µg/mL heparin was added, and the tubes were incubated with open caps for 6 hours at 38.5°C in an environment with 5% CO_2 and 85% relative humidity. After the incubation period, the semen samples were processed for acrosome reaction evaluation.

Assessment of acrosome reaction in presence of spermine-NONOate and genistein

Heparin-capacitated spermatozoa were treated with varying concentrations of the protein tyrosine kinase (PTK) inhibitor Genistein (1, 2, 3, and 4 µM) to determine the optimal concentration. After establishing the ideal dose, the spermatozoa were treated for 15 minutes with Spermine-NONOate, Genistein (3 µM), a combination of Spermine-NONOate and Genistein, and LPC (used as a positive control). The percentage of acrosome reaction (AR) was assessed by counting 200 cells following dual staining, as outlined by Suraj and Atreja (2000), to differentiate between physiological and degenerative acrosome loss. The smears were then examined under an oil immersion lens using bright field microscopy, with 200 cells per smear evaluated to assess the extent of the acrosome reaction.

Protein Tyrosine Phosphorylation in presence of Spermine-NONOate and Genistein

The freshly processed and diluted spermatozoa were incubated for 6 h in Sp-TALP

medium containing heparin (10 µg/mL). After incubation, the acrosome reaction (AR) was assessed by treating the heparin-capacitated sperm samples with or without Spermine-NONOate (100 µM), Genistein (3 µM), or a combination of both, followed by a 15-minute incubation in a CO_2 incubator. Sperm proteins were extracted using a modified protocol based on Galantino-Homer *et al*. (1997) for buffalo spermatozoa, and protein concentrations were determined using the Lowry method (1951). SDS-PAGE was carried out following Laemmli's method (1970). To detect tyrosine-phosphorylated proteins induced by nitric oxide in capacitated buffalo spermatozoa, an indirect immunoblotting technique was used. Antigens were transferred to a PVDF or nitrocellulose membrane, and nonspecific binding sites were blocked with skimmed milk. The membrane was then incubated with a monoclonal anti-phosphotyrosine antibody (clone pT-154; 1:2000) for 2 h at room temperature with gentle shaking. After brief (30 seconds × 2) and thorough (15 minutes × 4) washes with TBS-T, a secondary antibody (goat anti-mouse IgG; 1:2000) conjugated to HRP was applied. Chemiluminescence was used to visualize the peroxidase activity. Proteins separated by SDS-PAGE were transferred to an Immobilon-P PVDF membrane (0.45 µm) using a two-step transfer method (Otter *et al*., 1987). Coomassie Brilliant Blue-stained gels were photographed using a digital camera on a white light box, and X-ray films and CBB R-250 stained membranes were analyzed with an Alpha-Imager (Alpha-Innotech, USA). Relative mobility (Rf) values for each protein, including molecular weight markers, were calculated, and the band intensities were analyzed using Alpha Ease software, version FC 6.0.1.

Statistical analysis

All experiments were conducted at least three times, and data that adhered to a normal distribution were analysed using one-way ANOVA (analysis of variance). The results are presented as means ± S.E.M. Statistical differences between treatment groups were assessed using Duncan's Multiple Range Test (DMRT) with SPSS software, version 17.0.1 (SPSS Inc., Chicago, IL, USA). A P-value of less than 0.05 was considered statistically significant.

RESULT

Effect of spermine-NONOate and genistein on acrosome recation

As shown in Figure 1, spermine-NONOate significantly increased the percentage of acrosome reaction (AR) compared to the control group (41.07±1.79% vs. 19.09±1.38%, $P<0.05$). However, the addition of genistein to spermine-NONOate treatment led to a significant reduction in AR percentage (35.24±1.35% vs. 41.07±1.79%, $P<0.05$). Furthermore, treatment with lysophosphatidylcholine (LPC) alone significantly elevated AR compared to the control (51.36±1.03% vs. 19.09±1.38%, $P<0.05$).

Effect of spermine-NONOate and genistein on protein tyrosine phosphorylation during AR

Protein tyrosine phosphorylation levels were evaluated through immunoblotting of sperm proteins. A total of nine tyrosine-phosphorylated proteins-p20, p30, p32, p38, p45, p49, p69, p80, and p105 were identified, with varying intensities as determined by densitometric analysis (Figure 2 and Table 1). Phosphorylation was observed across proteins in the molecular weight range of 20 to

105 kDa, both in the presence and absence of PTK inhibitors and spermine-NONOate. Notably, p20 showed significantly higher phosphorylation in the spermine-NONOate-treated group compared to the spermine-NONOate + genistein group and was completely absent in samples treated with genistein alone.

In the spermine-NONOate-treated samples, p30 exhibited increased phosphorylation but was absent in both the genistein-treated group and the spermine-NONOate + genistein group, indicating complete loss of p30 following genistein treatment. Proteins p32, p38, p45, p49, p69, and p80 were present across all treatment groups, although their phosphorylation levels varied. Notably, p32, p45, p49, and p69 displayed significantly higher phosphorylation in the spermine-NONOate-treated group compared to the other groups, with a marked reduction upon genistein addition ($P<0.05$). Similarly, p38 showed significantly elevated tyrosine phosphorylation in the spermine-NONOate-treated group compared to the control, spermine-NONOate + genistein, and genistein-only groups ($P<0.05$). Both p80 and p105 also demonstrated significantly higher phosphorylation in the spermine-NONOate group, which was significantly reduced by genistein treatment ($P<0.05$).

DISCUSSION

Effect of spermine-NONOate and genistein on acrosome reaction

Genistein, a compound known for its estrogenic activity, also acts as a potent inhibitor of protein tyrosine kinase (PTK) (Nakashima *et al*., 1991). It has been shown to partially inhibit acrosomal loss induced by the nitric oxide donor SNAP by approximately 30%, with potential inhibition exceeding 90% under specific conditions. Genistein significantly suppresses progesterone- and ZP-3 to 6 peptide-mediated acrosome reaction (AR) induction, resulting in a dose-dependent reduction in sperm-zona binding, while having no effect on sperm motility or capacitation (Kirkman-Brown *et al*., 2002). Furthermore, treatment with PTK inhibitors has been reported to block exocytosis and sperm penetration of the zona pellucida triggered by both progesterone and the zona pellucida itself (Kirkman-Brown *et al*., 2002; Pukazhenthi *et al*., 1998; Menzel *et al*., 2007).

In our study, heparin-capacitated buffalo spermatozoa treated with spermine-NONOate exhibited a significant increase in acrosome reaction (AR), which was notably reduced by the addition of genistein (2 µM). These findings suggest that genistein effectively inhibits spermine-NONOate-induced AR, likely by modulating nitric oxide (NO) production. This observation aligns with the work of Leclerc *et al.* (1997), who demonstrated that genistein blocks AR induced by SNP, highlighting its role in the intracellular mechanisms driving NO triggered exocytotic events in capacitated spermatozoa. Additionally, genistein may also reduce capacitation, implying that endogenous $ONOO^-$ (peroxynitrite) is produced during heparin- or SNP-induced capacitation. Notably, exogenous $ONOO^-$ can act as a capacitation inducer, with PTK playing a critical role in the intracellular pathways involved in capacitation, as reported in cryopreserved bovine spermatozoa (Rodriguez and Beconi, 2009).

Effect of spermine-NONOate and genistein on protein tyrosine phosphorylation during AR

Mahony *et al.* (1999) reported that genistein did not affect hyperactivated motility in

Table 1. Relative Band Intensities (Mean ± SE) of Tyrosine Phosphorylated Proteins in Buffalo Spermatozoa in presence of Protein tyrosine kinase (PTK) inhibitor of AR.

Groups	Control (LPC)	Spermine-Nonoate	Genistein + Spermine-Nonoate	Genistein
p105	100	84.6217±1.4529^{a}	78.70961±1.5275^{b}	75.01413±1.4529^{c}
p80	100	96.05011±1.7638^{a}	84.34779±1.7320^{b}	74.16528±1.7638^{c}
p69	100	97.3821±1.7638^{ab}	93.68405±0.8819^{b}	81.20312±1.2018^{c}
p49	100	105.4795±0.5773^{a}	101.9787±1.7638^{a}	96.80365±1.1547^{c}
p45	100	96.66667±1.7320^{a}	87.46033±1.8559^{b}	82.38095±1.7320^{c}
p38	100	134.8781±2.0816^{a}	117.2979±1.2018^{b}	105.4816±1.1547^{c}
p32	100	113.9181±1.8559^{a}	75.23294±1.7638^{b}	76.30162±1.5275^{b}
p30	100	80.6541±1.1547^{a}	54.2039±1.2658^{b}	absent
p20	100	75.80024±0.8819^{a}	78.23933±0.5773^{b}	absent

Values are expressed as mean ± SEM from three independent samples. Different superscript letters ($^{a, b, c}$) indicate statistically significant differences (P<0.05).

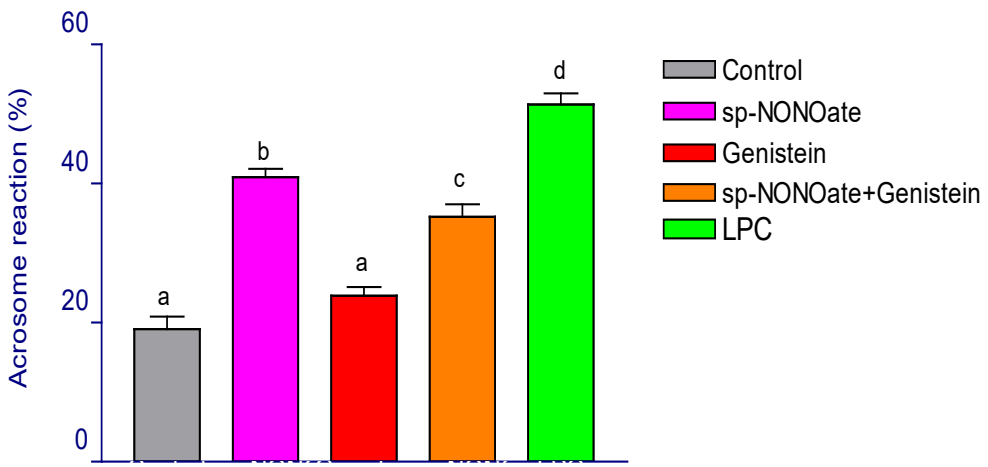

Figure 1. Effect of protein tyrosine kinase (PTK) inhibitor on acrosome reaction (AR). Heparin-capacitated buffalo spermatozoa were incubated for 15 minutes under various conditions: in the absence of any treatment (control), in the presence of spermine-NONOate (100 µM), genistein (3 µM), a combination of spermine-NONOate + genistein, and LPC (used as a positive control to induce AR). Data are presented as mean ± SEM from three independent samples. Different letters ($^{a, b, c, d}$) indicate significant differences (P<0.05).

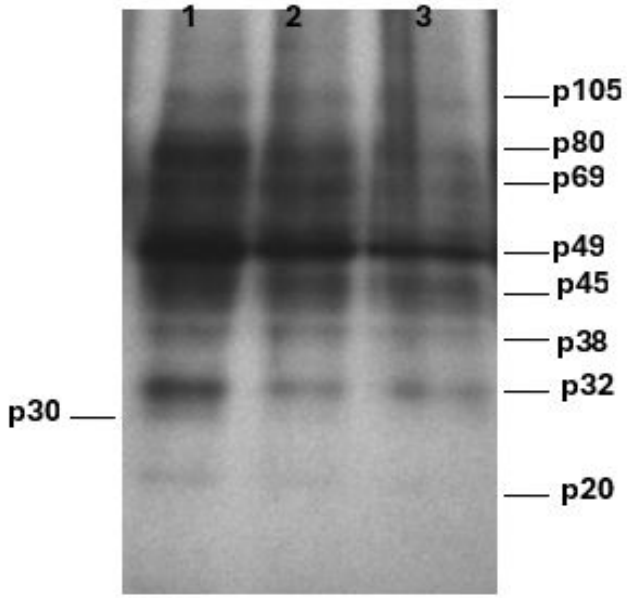

Figure 2. Effect of PTK inhibitor on protein tyrosine phosphorylation. Protein tyrosine phosphorylation during acrosome reaction in heparin capacitated buffalo spermatozoa in presence of PTK inhibitor (genistein). Lane 1, 2 and 3 represent spermine-NONOate, spermine-NONOate + genistein and genistein, respectively.

cynomolgus monkey spermatozoa in the absence of caffeine and dbcAMP, but significantly reduced caffeine- and dbcAMP-stimulated hyperactivation in a dose-dependent manner. In our study, heparin-capacitated buffalo spermatozoa were incubated with or without PTK inhibitors (genistein), spermine-NONOate, and a combination of both to induce the acrosome reaction (AR). Spermine-NONOate significantly ($P<0.05$) increased the phosphorylation of proteins p20, p30, p32, and p38. Notably, p20 and p30 showed higher phosphorylation in the spermine-NONOate group but were absent in the genistein-treated samples. Proteins p32, p38, p45, p49, p69, and p80 were present across all treatment groups, although their phosphorylation levels varied. These results are consistent with previous findings (Leyton and Saling, 1989; Tesarik *et al*., 1996; Baldi *et al*., 2000), which demonstrated PTK involvement in the phosphorylation of p80 and p105. Nitric oxide (NO) can directly activate PTK, as shown in various cell types (Bauskin *et al*., 1991; Yoshida *et al*., 1999). Furthermore, PTK inhibitors such as tyrphostin A47, genistein, and lavendustin have been shown to block progesterone-induced AR in human sperm (Luconi *et al*., 1995; Meizel and Turner, 1993; Kirkman-Brown *et al*., 2002). Additionally, LPC- and A23187-induced AR are associated with increased tyrosine phosphorylation of p80 and p105 (Aitken *et al*., 1995; de Lamirande *et al*., 1998; de Lamirande and Gagnon, 2002).

Protein kinase A (PKA), a serine/threonine kinase, does not directly phosphorylate tyrosine residues but can indirectly activate tyrosine kinases (Leclerc *et al*., 1996). Protein tyrosine kinase (PTK) is essential for the phosphorylation of tyrosine

residues in proteins involved in capacitation (Leclerc *et al*., 1996). The observed reduction in acrosome reaction (AR) with specific PTK inhibitors suggests that PTK plays a critical role in NO-induced AR. Activation of PTK may result from PKA-mediated phosphorylation (Rodriguez *et al*., 2005), a mechanism also reported in human spermatozoa (Leclerc *et al*., 1997). Therefore, PTK is crucial for enhancing protein tyrosine phosphorylation during the acrosome reaction in buffalo spermatozoa.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the National Dairy Research Institute, Karnal, the Indian Council of Agricultural Research, and the Director of NDRI, India, for their financial and logistical support in conducting this research.

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