Indian Journal of Animal Sciences **94** (12): 1096–1099, December 2024/Short communication
https://doi.org/10.56093/ijans.v94i12.153327

Characterization of membrane-associated non-genomic progesterone receptors in cattle bull spermatozoa

VIKAS SACHAN[1]✉, SOUMEN CHOUDHURY[2], DILIP K SWAIN[2], ANUJ KUMAR[2], ATUL SAXENA[2], PRATISHTHA SHARMA[3] and AVANEESH KUMAR SINGH[4]

UP Pandit Deen Dayal Upadhaya Pashuchikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan, Mathura, Uttar Pradesh 281 001 India

Received: 1 July 2024; Accepted: 13 November 2024

Keywords: Cattle, Immunolocalization, Non-genomic receptors, Progesterone, Spermatozoa

After deposition in the female genitalia, sperm cells interact with uterine and oviductal secretions and as the time of ovulation approaches, sperms begin to hyperactivate, i.e. undergo complex biomorphological changes; capacitation. Capacitation is an intricate mechanism which involves numerous pathways (Fraser 2010) with the remarkable event being a rise in protein tyrosine phosphorylation (Breitbart 2003). Protein kinase A (PKA), HCO_3^-, soluble adenylate cyclase (SACY or sAC), cyclic adenosine 3'5'monophosphate (cAMP), etc are the factors that control the modifications during capacitation (Visconti 2009). Capacitation in spermatozoa makes it capable of attaching with the extracellular matrix of the oocyte which is essential for the acrosome reaction to ensure the fertilization within a specific time frame. Progesterone is the steroid present in the follicular fluid that induces both human sperm motility and acrosome response (Calogero *et al*. 2000). Progesterone has the ability to initiate the acrosome reaction *in vitro* in several mammalian sperms, including caprines (Somanath and Gandhi 2000) and canines (Brewis *et al*. 2001).

Steroids often operate through cellular receptors and take days or even longer to exhibit their effects but in contrast, progesterone, a steroid, causes transcriptional biomorphological changes in sperm within minutes, including capacitation and acrosome response. Actually, rather than the classical intracellular progesterone receptors (PR), progesterone acts through non-genomic surface progesterone receptors in the case of spermatozoon. Non-genomic membrane progesterone receptor was proposed (Contreras and Llanos 2001) which was discovered to be located in the acrosomal area of human spermatozoa. It was interesting to investigate whether the non-genomic surface receptors for progesterone are present in bull spermatozoa and what is their location. This study, investigated the existence of non-genomic progesterone receptors, their localization as well as their molecular characterization in bull spermatozoa.

Reagents and chemicals: Chemicals and reagents used during the study were of biological grade and were purchased by Sigma Aldrich, St Louis, MO, USA unless mentioned.

Semen collection: A total of 64 semen ejaculates were obtained from four Hariana cattle bulls (twice a week from each bull using an artificial vagina) kept in a semi-intensive management system at the Institutional Livestock Farm Complex (ILFC) of the University. The obtained semen samples were immediately taken to the laboratory for further examination and preparation for *in vitro* research.

Evaluation of semen: Collected semen ejaculates initially underwent some basic quality evaluations. Fresh semen was assessed for mass motility of spermatozoa, *p*H, Spermatozoa concentration and sperm viability by microscopic examination, *p*H paper, CASA and Eosin-Nigrosin staining, respectively.

Identification of non-genomic progesterone receptors: The qualified and screened semen samples were processed for *in vitro* investigation of the existence of progesterone receptors.

mRNA expression of membrane progesterone receptor (Non-genomic P4 receptor) by RT-PCR: Sperm samples collected from four bulls were used for isolation of RNA. cDNA production was done from the mRNA available with the help of Revertaid® First strand cDNA synthesis kit (Thermo Scientific, USA) using Moloney murine leukaemia virus reverse transcriptase enzyme as per manufacturer's instructions. Using the reference sequences of these receptors for bovine and caprine published in the NCBI and Ensemble data bank, the primer sequences were designed for amplification of these receptors in bovine sperm cells

Present address: [1]COVAS, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, Uttar Pradesh. [2]COVSc & AH, UP Pandit Deen Dayal Upadhaya Pashuchikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan, Mathura, Uttar Pradesh. [3]Lighthouse Community Charter School, Hegenberger Rd, Oakland 94621, California, USA. [4]International Institute of Veterinary Education and Research, Rohtak, Haryana. ✉Corresponding author email: vikas.vet23@gmail.com

Table 1. Description of primers

Gene	Primer sequence	Amplicon size (bp)	Annealing Temp. (°C)
mPR	F5′-GAGAGCACTCGGAAGAATGATTA-3′ R5′-TGCAGTTAGGTCAATCGAGAAA-3′	466	59.0

using DNA STAR, Gene Tool and Primer Quest software (Table 1). The RT-PCR mixture composition included 6.25 µL of PCR master mix (2X), 0.5 µL of forward primer (10 pmol/µL), 0.5 µL of reverse primer (10 pmol/µL), 1.0 µL of template, and nuclease-free water to a total volume of 12.5 µL. The thermal cycling conditions were: Initial denaturation at 95ºC for 5 min, Denaturation at 95ºC for 1 min, Annealing at 55ºC to 60ºC for 1 min, Extension at 72ºC for 1 min (Repeat steps 2-4 for 40 cycles), Final extension at 72ºC for 5 min, and finally held at 4ºC.

The cDNA was assessed for quality by running PCR with standard GAPDH primers. Then, amplified DNA was subjected to agarose gel electrophoresis as detailed by Sambrook and Russel (2001). The DNA samples were visualized as bands under UV light and documented. The size of the amplified PCR product was determined from the standard 100 bp DNA ladder (Invitrogen, USA).

Immunoblotting of sperm proteins for progesterone receptor: Sperm protein was obtained by the complete protein cell lysate isolation kit (Amresco, USA). For protein isolation, SDS page technique was adopted with standard chemicals and gels (Supplementary Table 1). Western blotting was performed for the detection of proteins corresponding to progesterone receptors. Isolated proteins were quantified by using commercial kit (Gennei, Merck, India). Primary antibody for progesterone receptors (PA5/42919 rabbit polyclonal antibodies against progesterone receptors, Thermofisher scientific) was used in the study. The antibody dilution was carried out at (1:150) in TBST. Secondary antibody A-6154 Anti rabbit HRP conjugate (Sigma, St Louis) diluted (1:5000) in TBST was used.

Development of the protein bands was carried out by using DAB system (SIGMAFAST DAB tablets, St Louis, USA). The relative molecular weights were assessed with the help of the broad-range molecular weight markers (Color Prestained Protein Standard, Broad Range New England Biolabs / Prestained colour biolabs protein molecular weight marker) and Gel documentation and analysis system (Gel- Doc. Model-Alpha Imager TM1220; Alpha Innotech Corporation, Santa Clara, CA, USA).

Immunolocalisation of progesterone receptors: The immunolocalisation of progesterone receptors was carried out by performing an indirect immunofluorescence technique (Chauhan *et al*. 2017). The technique employed antibodies to localize the progesterone receptor proteins on the sperm surface. Primary antibodies (PA5/42919, Rabbit polyclonal against progesterone receptors, Sigma, St Louis USA, diluted (1:50) in TBST) and secondary antibody (F-0382, Anti-rabbit FITC conjugate, Sigma, St Louis, diluted (1:1000) in TBST) were used for the purpose. After the use of antifading medium, slides were observed using Nikon Eclipse TE 2000-S microscope with phase contrast and epifluorescence optics under blue-violet illumination (excitation at 400–440 nm and emission at 470 nm by using 60× objective) using blue / FITC filter. A total of 200 sperm cells on each slide were examined, and distinct spermatozoa distribution patterns were assessed based on the site of fluorescence on the sperm.

mRNA expression of membrane progesterone receptor (Non-genomic P4 receptor) by RT-PCR: In the study, the molecular presence of progesterone receptors (PRs) in bull spermatozoa was confirmed through isolated mRNA and its amplification using designed and specific primers. Amplification of membrane progesterone receptor using gene-specific primers showed RT-PCR product of 466 bp length as expected from the software-based analysis, thus confirming the presence of membrane progesterone receptor gene in bull spermatozoa (Fig. 1). Similar to the present study, Shah *et al.* (2003) revealed the existence of mRNA for PRs in human spermatozoa, however, there is no investigation on the identification of non-genomic PR in animals based on isolation and amplification of mRNA.

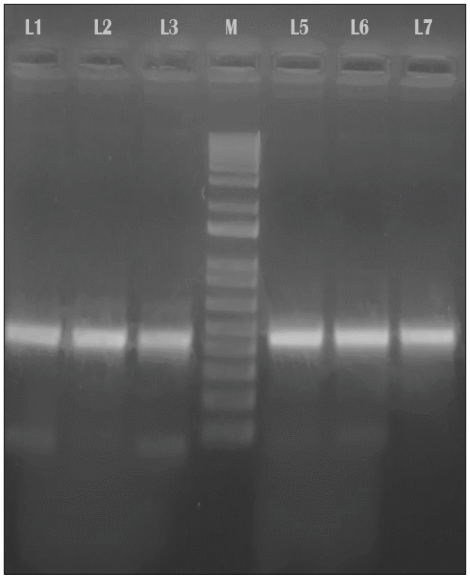

Fig. 1. mRNA expression of membrane progesterone receptor [M: 100 bp Ladder; L1-L7: *mPR* (466 bp)].

Immunoblotting of sperm proteins for progesterone receptor: Further, the validation of the molecular presence of PRs in bull spermatozoa was confirmed through immunoblotting studies. Western blotting was performed for the detection of protein corresponding to progesterone receptors and the corresponding results are summarised in Fig. 2. Immunoblotting reported a protein of ~56 kDa matching PR in cattle bull spermatozoa. In the study, a polyclonal antibody was used against the PR

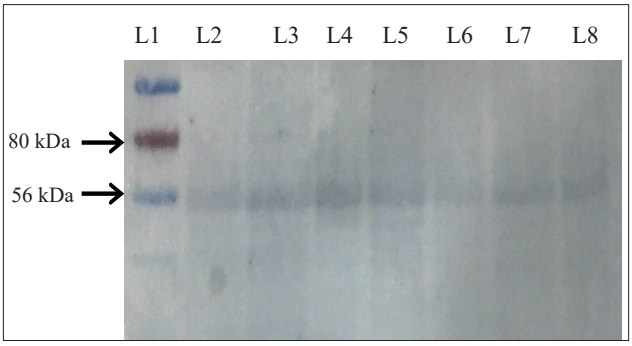

Fig. 2. Immunoblotting of sperm lysate proteins showing single band of MW 56 kDa pertaining to non-genomic progesterone receptor in bull spermatozoa (L1: MW marker; L2, L3, L4 and L5: Bull sperm lysates).

protein, however, a single reactive band in the blotting experiments confirmed the presence of PR. In the study, the use of polyclonal antibodies raises the possibility of multiple reactive bands, however, a single reactive band was formed. These results evidently suggested that membrane progesterone receptors are present in *Hariana* bull spermatozoa. Non-genomic progesterone receptors in spermatozoa of boar were also observed by western blotting from the membrane but not the cytoplasm by Jang and Yi (2005). Luconi *et al.* (2002) documented two separate proteins with molecular weights of 57 and 28 kDa using mAb C-262 as potential membrane PR in human sperm cells.

Immunolocalisation of progesterone receptors: Further, confirmation of the molecular presence of surface PRs in bull spermatozoa was done through immunolocalisation studies. Indirect immune fluorescence results are illustrated in Fig. 3. Results showed positive immune reactivity to antibody binding, leading to confirmation of the existence of progesterone receptors on bull spermatozoa. Immunofluorescence showed maximum positive interaction at the acrosome and post-acrosomal area of the sperm in bull. Binding was observed to be heterogeneous in groups

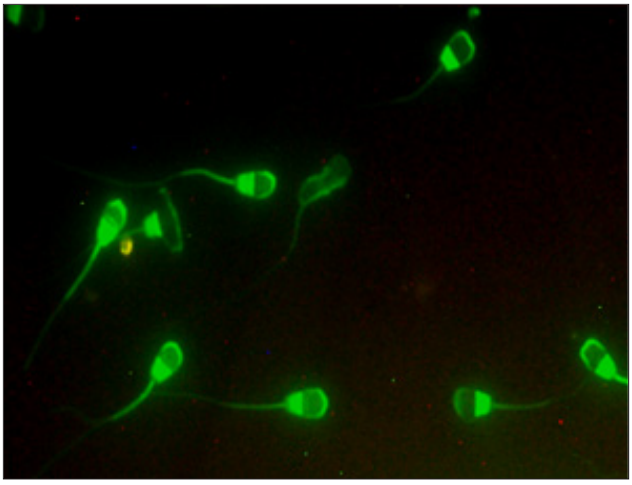

Fig. 3. Immunolocalisation of progesterone receptors in bull spermatozoa (60×) at the acrosomal and post acrosomal region.

of ejaculated spermatozoa indicating a mixed population of sperm cells in ejaculated semen. Similarly, P-BSA-FITC or a monoclonal antibody against the progesterone receptor and secondary FITC-conjugated antibodies were used to investigate the progesterone receptor's location on stallion spermatozoa and they were found at the acrosomal area. Similarly, Somanath and Gandhi (2002) found membrane PRs at the apical section of the acrosome and the post-acrosomal region in all caprine sperm based on P–BSA–FITC conjugate binding studies. Non-genomic surface PR was hypothesised and found to be located in the acrosomal region (Wu *et al*. 2005) in human spermatozoa. Modi *et al.* (2007) also confirmed the non-genomic receptors localized on the sperm acrosome as evident from *in situ* hybridization techniques in human spermatozoa.

Results of PCR-based detection of mRNA, a product of 466 bp confirmed the presence of membrane progesterone receptors. Immunoblotting showed a protein of 56 kDa pertaining to the non-genomic progesterone receptors in bull spermatozoa. Immunofluorescence and Immunolocalization demonstrated the presence of progesterone receptors in *Hariana* bull spermatozoa at the acrosome and post-acrosomal region.

SUMMARY

The present study aimed to examine the molecular existence of the non-genomic progesterone receptors (P4) and their location in *Hariana* bull spermatozoa. The investigation involved the collection and processing of 64 semen ejaculates from four *Hariana* bulls. Following spermatozoa protein isolation, immunoblotting revealed the presence of 56 kDa protein corresponding to progesterone (P4) membrane receptors, and the results were validated using RT-PCR, which indicated an amplicon of 466 bp. Immuno-fluorescence technique was used to confirm the location of P4-membrane receptors in spermatozoa. The present study revealed the presence of 56 kDa protein representing progesterone receptors at the acrosome as well as post-acrosomal region of *Hariana* cattle bull spermatozoa.

ACKNOWLEDGEMENTS

The authors thank the administrative authorities, U.P. Pt. Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya, Mathura for giving permission and providing the necessary facilities to conduct this work. Thanks are extended to the Head of the Department of Veterinary Physiology and Veterinary Pharmacology and Toxicology for their support with techniques and instrumentation while working.

REFERENCES

Breitbart H. 2003.Signaling pathways in sperm capacitation and acrosome reaction. *Cell and Molecular Biology* **49**(3): 321–27.

Brewis I A, Morton I E, Moore H D and England G C. 2001. Solubilized zona pellucida proteins and progesterone induce calcium influx and the acrosome reaction in capacitated dog spermatozoa. *Molecular Reproduction and Development*

60(4): 491–97.

Calogero A E, Burrello N, Barone N, Palermo I, Grasso U and D'Agata R. 2000. Effects of progesterone on sperm function: Mechanisms of action. *Human Reproduction* **15**(1): 28–45.

Chauhan D S, Swai D K, Shah N, Yadav H P, Nakade U R, Singh V K, Nigam R, Yadav S and Garg S K. 2017. Functional and molecular characterization of voltage-gated sodium channel Nav 1.8 in bull spermatozoa. *Theriogenology* **90**: 210–18.

Contreras H R and Llano M N. 2001. Detection of progesterone receptors in human spermatozoa and their correlation with morphological and functional properties. *International Journal of Andrology* **24**(4): 246–52.

Fraser L R. 2010. The "switching on" of mammalian spermatozoa: molecular events involved in promotion and regulation of capacitation. *Molecular Reproduction and Development* **77**(3): 197–208.

Jang S and Yi L S H. 2005. Identification of a 71 kDa protein as a putative non-genomic membrane progesterone receptor in boar spermatozoa. *Journal of Endocrinology* **184**(2):417–25.

Luconi M, Bonaccorsi L, Bini L, Liberatori S, Pallini V, Forti G and Baldi E. 2002. Characterization of membrane nongenomic receptors for progesterone in human spermatozoa. *Steroids* **67**(6): 505–09.

Modi D, Shah C A and Puri C P. 2007. Non genomic membrane progesterone receptors on human spermatozoa. *Society for Reproduction and Fertility* **63**: 515–29.

Sambrook J and Russell D W. 2001. *Molecular Cloning: A Laboratory Manual*, 3 edn, pp. 2100. Cold Spring Harbor Laboratory Press, New York.

Shah C A, Modi D, Gadkar S, Sachdeva G and Puri C P. 2003. Progesterone receptors on human Spermatozoa. *Indian Journal of Experimental Biology* **41**(7): 773–80.

Somanath P R and Gandhi K K. 2002. Expression of membrane associated non-genomic progesterone receptor(s) in caprine spermatozoa. *Animal Reproduction Science* **74**(3-4): 195–205.

Visconti P E. 2009. Understanding the molecular basis of sperm capacitation through kinase design. *Proceedings of the National Academy of Sciences, USA* **106**(3): 667–68.

Wu J T, Tsai P S, Lee S L and Cheng F P. 2005. Characterisation of the progesterone receptor on canine spermatozoa. *Reproduction, Fertility and Development* **17**(7): 733–41.