*Indian Journal of Animal Sciences* **92** (8): 946–949, August 2022/Article
https://doi.org/10.56093/ijans.v92i8.120018

# Asthenozoospermic stallions tend to have high acrosome reacted spermatozoa as evidenced by dual fluorescent staining assay

KATHAN RAVAL[1], NILENDU PAUL[1], PRADEEP NAG[1], ELANGO K[1], YASH PAL[2], LEGHA R A[2], T R TALLURI[1]✉ and ARUMUGAM KUMARESAN[1]

*Southern Regional Station, ICAR-National Dairy Research Institute, Bengaluru, Karnataka 560 030 India*

Received: 8 January 2022; Accepted: 11 July 2022

ABSTRACT

Acrosome intactness of spermatozoa is the critical factor for establishing sperm reservoir in oviduct and for fertilizing an oocyte. However, frozen thawed spermatozoa tend to show higher proportion of acrosome reacted spermatozoa thereby compromising the fertility. Conventional staining techniques identify only sperm acrosome integrity and not precisely the acrosome reaction status. In this context, the current study was conducted to assess the acrosome status of cryopreserved spermatozoa using Fluorescein isothiocyanate conjugated peanut agglutinin and propidium iodide (FITC-PNA+PI) in stallions with varying sperm motility. Stallions were classified into high- (≥45%) and low-motile group (≤30%) based on their post-thaw sperm motility. The proportion of live acrosome intact (LAI) spermatozoa was significantly higher in high-motile group as compared to low-motile group. A significant positive correlation was observed between LAI and post-thaw sperm motility. In conclusion, the present study showed that FITC-PNA+PI combination could be used for rapid and accurate assessment of acrosome reaction status of stallion spermatozoa, and the proportion of LAI population in cryopreserved stallion semen had a strong correlation with sperm motility.

**Keywords**: Acrosome reaction, FITC-PNA, Motility, Propidium iodide, Stallion spermatozoa, Viability

The equine breeding industry primarily relies on cryopreserved stallion sperm for rapid propagation of superior germplasm. However, equine semen cryopreservation is still at infancy as compared to the use of fresh and chilled semen (Talluri *et al.* 2019, Aurich *et al.* 2020). Freezing of equine semen aids in extending the survival of spermatozoa for later use. On the contrary, the process of freezing and thawing leads to sperm damage, especially to the acrosome that is important for fertilizing oocyte thereby reducing the chances of conception when cryopreserved semen is used for breeding. Physiologically, in acrosome reaction (a pre-requisite for fertilization), acrosomal contents are released as a result of fusion of plasma membrane of spermatozoa with outer acrosome membrane, that allows adhesion and penetration of spermatozoa to zona pellucida and gamete fusion (Gerton 2002). Interestingly, cryopreserved semen also shows pre-mature acrosome reaction that reduces the longevity of the spermatozoa in female reproductive tract and sperm fertilizing potential, and the extent of such damage varies with species. Therefore, assessment of the status of sperm acrosome reaction in a given semen sample assumes significance in artificial breeding.

Present address: [1]Southern Regional Station, ICAR-National Dairy Research Institute, Bengaluru, Karnataka. [2]ICAR-National Research Centre on Equines, Hisar, Haryana. ✉Corresponding author email: raotalluri79@gmail.com

Traditionally, sperm acrosomal integrity is evaluated by Giemsa stain (Watson 1975) which can differentiate only two sperm populations i.e. acrosome intact and acrosome damaged spermatozoa. Further, comparatively smaller size of acrosome in stallion makes it difficult to evaluate using traditional staining methods. Recently, combination of Fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) and propidium iodide (PI) is shown to assess different status of sperm acrosome and viability.

With this backdrop, in the present study, we assessed the sperm acrosome reaction status using a combination of FITC-PNA and PI in semen of stallions with varying sperm motility. We hypothesized that stallions with variable sperm motility might have different sperm populations in terms of acrosome reaction status.

## MATERIALS AND METHODS

The present experiment was conducted at Theriogenology Laboratory of Southern Regional Station of ICAR-NDRI, Bengaluru, Karnataka, India. Stallions were classified into high motile group (n=3; post-thaw progressive motility ≥45%) and low motile group (n=3; post-thaw progressive motility ≤30%) based on post-thaw sperm motility (Fig. 1A).

*Semen collection and cryopreservation:* Semen was collected from 6 adult stallions of age 4 to 6 years being maintained at Equine Production Centre, ICAR-National

Research Centre on Equines, Bikaner, Rajasthan, India. No separate provisions were made for light or feed for the stallions involved in study. Semen was collected early in the morning and estrus mare was used as a dummy. Semen collection was done using Colorado model artificial vagina (AV) which was lubricated with liquid paraffin and pre-warmed to 45-50ºC and fitted with an inline filter to separate the gel fraction. The semen was collected over a four-day period before to the start of the actual experiment. Two days of sexual rest was given to the stallions before the starting of the experiment. Each stallion was given one false mount before actual collection. Thereafter, two ejaculates were collected from each stallion. Soon after collection, ejaculates were transported to the laboratory and kept at 37ºC until processing. Sperm motility, concentration parameters were evaluated and six stallions having total motility >50-60% were selected for the study. In order to remove the seminal plasma, semen sample were centrifuged at 650 g for 5 min and supernatant was discarded. The sperm pellet was extended in secondary freezing extender (6 g glucose, 11 g lactose, 0.37 g EDTA, 0.37 g sodium citrate, 0.12 g sodium bicarbonate, 10% egg yolk, 100,000 IU penicillin, and 0.10 g streptomycin) containing 5% dimethyl formamide as cryoprotectant. The extended semen ($150\times10^6$ sperms/mL) was loaded into 0.5 mL straws (IMV-Technologies, France) and the straws were sealed with an automatic filling and sealing machine (IMV-Technologies, L'Aigle, France). Further, filled straws were equilibrated at 4ºC for 2 h. After equilibration, freezing of the stallion semen was performed by customized method of freezing in liquid nitrogen vapours by spreading the straws on a straw stand at height of 4 cm and then the straws were taken out after 12 min exposure and plunged into canisters of liquid nitrogen (–196°C) containers till further analysis.

*Assessment of acrosomal status of stallion spermatozoa:* Cryopreserved semen from all the 6 stallions was thawed at 37°C for 30 sec and spermatozoa were suspended in sperm TALP (spTALP) (3.1 mM KCl, 100 mM NaCl, 0.29 mM $NaH_2PO_4$, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 21.6 mM $C_3H_5NaO_3$ and 1.5 mM $MgCl_2$). This sperm suspension was then washed two times by centrifuging at 300 g for 3 min to remove the diluent. Sperm acrosome status was evaluated using combination of FITC-PI as described by Nag *et al.* (2021). Briefly, after washing of spermatozoa with spTALP, concentration of spermatozoa was set to $2 \times 10^6$ in spTALP to make the final volume of 200 µL. After washing, 1 µL of FITC-PNA (1 mg/mL) was added to the spermatozoa and incubated in the dark for 10 min at 37ºC. PI (2 µL; 2.4 mM) was then added to sperm suspension and further incubated for 2 min. Later, sperm suspension was centrifuged and supernatant was discarded, thin smear was made from the sperm pallet on the grease free glass slide. After drying the smear, an antifading agent [1,4-diazabicyclo [2.2.2] octane (DABCO)] was added and a cover slip was placed over the smear. Spermatozoa was evaluated by inverted fluorescent microscope (Nikon ECLIPSE Ti-s, Japan) in FITC (Emission-515-555 and Excitation-465-495) and TRITC filter (Emission-554-576 and Excitation-540) at 1000× magnification. Images from both the filters were later merged to obtain the final image. A minimum of 200 spermatozoa/ per slide were counted and different subpopulations were evaluated. Four different sperm subpopulations were identified, viz. live acrosome intact (LAI), live acrosome reacted (LAR), dead acrosome intact (DAI) and dead acrosome reacted (DAR). The representative photograph of each subpopulation is shown in Fig. 1B.

*Statistical analysis:* Student's t-test was done to assess the differences in sperm acrosome integrity status between high- and low-motile stallion groups. Pearson correlation was performed between % post-thaw motility (PTM) and different subpopulation of spermatozoa in term of acrosome status to evaluate the degree of linear relationships between the parameters. The difference was considered significant when $p<0.05$. All the statistical analysis were performed using *GraphPad* Prism version 8.4.3.

## RESULTS AND DISCUSSION

PTM of semen was evaluated immediately after thawing of semen straws. PTM of high- and low-motile group was 47.73±1.12 and 24.78±1.95 (Mean±SEM), respectively. A significant difference ($p<0.05$) in motility was observed between high- and low-motile groups (Fig. 1A). Dual-staining method to assess the acrosome status of spermatozoa identified four different population

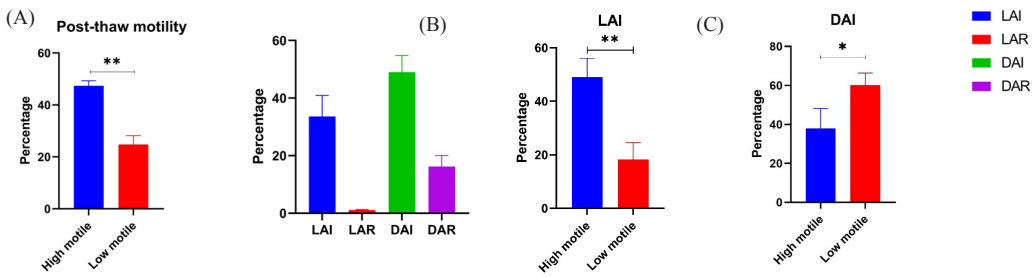


Fig. 1. (A) Percentage post-thaw sperm motility in high- and low-motile group stallions. (B) Proportion of different sub-populations of stallion spermatozoa in terms of acrosome status using FITC-PNA & PI. (C) Proportional difference in LAI and DAI spermatozoa sub-populations between high- and low-motile stallions.

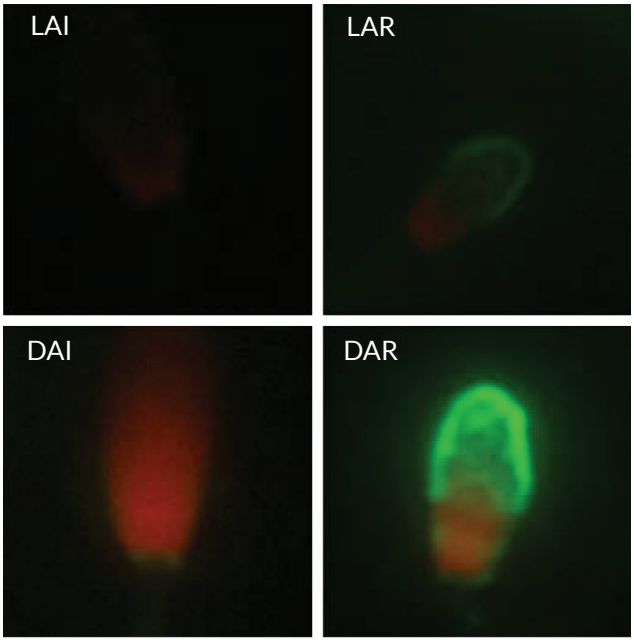


Fig. 2. Different sub-populations of stallion spermatozoa in terms of acrosome status assessed using FITC-PNA & PI.

of spermatozoa (Fig. 2) and proportion of different sub-population in terms of acrosome status is shown in Fig. 1B. In stallions from high-motile group, the proportion of LAI spermatozoa was significantly higher (P=0.0048) and DAI spermatozoa was significantly lower ($p<0.05$) in comparison to stallions of low-motile group (Fig. 1C). There was no significant difference in the proportion of LAR spermatozoa and DAR spermatozoa between high- and low-motile group.

Pearson correlation between %PTM and different subpopulations of spermatozoa in terms of acrosome status revealed that %PTM was positively correlated with LAI spermatozoa and negatively correlated with LAR and DAI spermatozoa of stallions belongs to high-motile group (Fig. 3).

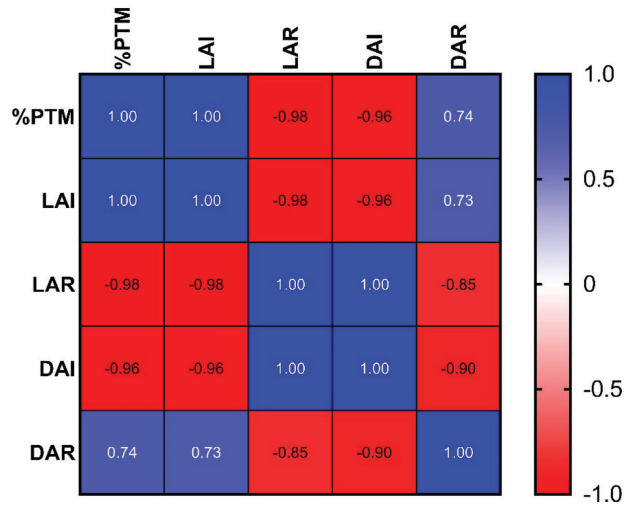


Fig. 3. Pearson correlation between %post-thaw motility and different sub-population of spermatozoa in term of acrosome status in high-motile group.

It is prerequisite to evaluate the semen before artificial insemination to ensure supply of the quality spermatozoa for successful conception. *In vivo*, before fertilization, sperm plasma membrane fuses with the acrosomal membrane at multiple sites and acrosome contents are released. These contents are involved in dissolving the zona-matrix and facilitates the sperm to enter the perivitelline space (Gadella *et al.* 2001). However, in premature acrosome reaction that occurs during freezing and thawing, the acrosomal contents will be lost and the fertilising ability of the spermatozoa will be compromised. Thus, for an oocyte to be fertilized, it is necessary that acrosome reaction takes place in spermatozoa at right time (Hirohashi and Yanagimachi 2018). The equine spermatozoa are highly susceptible to cryopreservation induced damages because of higher proportion of polyunsaturated fatty acid (PUFA) on their plasma membrane resulting into greater production of ROS (Aurich *et al.* 2018) that alters the acrosome status (Kumar *et al.* 2020). It has been reported that during cryopreservation, live acrosome intact spermatozoa will be converted to live acrosome reacted or dead acrosome reacted spermatozoa (Paudel *et al.* 2010).

We observed higher proportion of LAI spermatozoa in stallions of high-motile group as compared to that of low-motile group. Results are in agreement with earlier studies (Singh *et al.* 2016, Kumaresan *et al.* 2017), which reported that proportion of live acrosome intact spermatozoa was higher in high fertile bulls. It has been reported that spermatozoa with intact acrosome are being selected for establishment of oviduct reservoir (Gualtieri and Talevi 2000, Saraf *et al.* 2019) and can penetrate the zona pellucida ultimately resulting in successful fertilization. However, premature acrosome reaction is associated with idiopathic male infertility (Esteves and Verza 2011). Therefore, evaluation of sperm acrosome status might enhance the fertilization success with frozen-thawed equine semen.

Also, in the present study, a high positive correlation of LAI spermatozoa with % PTM was observed. This finding can be justified as hyper-activated motility is prerequisite for sperm penetration into the intact oocyte-cumulus complexes and fertilization (Yanagimachi and Bhattacharyya 1988). The acrosome reacted spermatozoa with lower motility fail to establish sperm reservoir in oviduct epithelium prior to ovulation (Bosch and Wright 2005). Hence, motile and acrosome intact spermatozoa are indispensable for fertilization. This can be one of the important factors behind lower conception rate in mares when spermatozoa with higher %acrosome reacted are used for insemination. Thus, incorporation of fluorescent dye-based approaches for the simultaneous detection of various sperm functional quality parameters like acrosome integrity and viability will help in identifying a good semen sample for artificial insemination to achieve higher conception rates.

A live spermatozoon with intact acrosome is mandatory for successful fertilization, hence it is important to evaluate the frozen semen for the same. Since, the conventional

methods to assess the acrosomal membrane status are not efficient enough to identify different sperm sub-populations that influences the fertilizing potential of spermatozoa, incorporation of dual fluorescent staining would not only save the time but also provide valuable information about the semen quality.

Cryopreservation of stallion semen is challenging and requires more attention as compared to bull. The post-thaw quality of stallion semen tends to show poor quality due to their higher degree of cryo-susceptibility. Thus, it is need of the hour to assess the post-thaw semen quality using advanced techniques so to make sure that inseminated semen quality might result in better success when introduced in a fertile mare. Fluorescent dye-based semen assessment is thus recommended to assess vital sperm functional parameters prior to use in artificial insemination.

## ACKNOWLEDGEMENTS

The authors are highly thankful to the Director, ICAR-National Research Centre on Equines, Hisar, India and Director, ICAR-National Dairy Research Institute, Karnal, India for providing the necessary facilities and infrastructure to conduct the present study.

## REFERENCES

Aurich C, Ferrusola C O, Vega F J P, Schrammel N, Morcuende D and Aurich J. 2018. Seasonal changes in the sperm fatty acid composition of Shetland pony stallions. *Theriogenology* **107**: 149–53.

Aurich J, Kuhl J, Tichy A and Aurich C. 2020. Efficiency of semen cryopreservation in stallions. *Animals* **10**(6): 1033.

Bosch P and Wright Jr R. 2005. The oviductal sperm reservoir in domestic mammals. *Archivos de Medicina Veterinaria* **37**(2): 95–105.

Esteves C S and Verza Jr S. 2011. Relationship of *in vitro* acrosome reaction to sperm function: an update. *Open Reproductive Science Journal* **3**(1).

Gadella B, Rathi R, Brouwers J, Stout T and Colenbrander B. 2001. Capacitation and the acrosome reaction in equine sperm. *Animal Reproduction Science* **68**(3-4): 249–65.

Gerton G L. 2002. Function of the sperm acrosome. *Fertilization.* pp 265-302. Elsevier.

Glazar A I. 2014. Assessment of sperm acrosomal status: FITC PNA. *Equine Reproductive Procedures* **483**: 476-77.

Gualtieri R and Talevi R. 2000. *In vitro* cultured bovine oviductal cells bind acrosome-intact sperm and retain this ability upon sperm release. *Biology of Reproduction* **62**(6): 1754–62.

Hirohashi N and Yanagimachi R. 2018. Sperm acrosome reaction: its site and role in fertilization. *Biology of Reproduction* **99**(1): 127–33.

Kumar P, Kumar A, Mehta J, Purohit G, Ravi S, Pal Y, Legha R, Tripathi B and Talluri T. 2020. Impact of supplementation of semen extender with antioxidants on quality of cooled or cryopreserved Marwari stallion spermatozoa. *Indian Journal of Animal Sciences* **90**(10): 1356-61.

Kumaresan A, Johannisson A, Al-Essawe E M and Morrell J M. 2017. Sperm viability, reactive oxygen species, and DNA fragmentation index combined can discriminate between above-and below-average fertility bulls. *Journal of Dairy Science* **100**(7): 5824–36.

Nag P, Kumaresan A, Sivamanikandan A, Manimaran A, Rajendran D, Paul N, Sharma A, Karuthadurai T, Kaustubh S and Jeyakumar S. 2021. Sperm phenotypic characteristics and oviduct binding ability are altered in breeding bulls with high sperm DNA Fragmentation index. *Theriogenology* **172**: 80–87.

O'Flaherty C, Beorlegui N and Beconi M. 2001. Role of superoxide anion and hydrogen peroxide in acrosome reaction of bovine spermatozoa. *Andrology in the 21st Century.* pp 103-108.

Paudel K, Kumar S, Meur S and Kumaresan A. 2010. Ascorbic acid, catalase and chlorpromazine reduce cryopreservation-induced damages to crossbred bull spermatozoa. *Reproduction in Domestic Animals* **45**(2): 256–62.

Saraf K K, Singh R K, Kumaresan A, Nayak S, Chhillar S, Lathika S, Datta T K and Mohanty T K. 2019. Sperm functional attributes and oviduct explant binding capacity differs between bulls with different fertility ratings in the water buffalo (*Bubalus bubalis*). *Reproduction, Fertility and Development* **31**(2): 395–403.

Singh R K, Kumaresan A, Chhillar S, Rajak S K, Tripathi U K, Nayak S, Datta T, Mohanty T and Malhotra R. 2016. Identification of suitable combinations of *in vitro* sperm-function test for the prediction of fertility in buffalo bull. *Theriogenology* **86**(9): 2263–71.

Talluri T, Ravi S, Singh C, Legha R, Mehta S, Prakash V and Tripathi B. 2019. Effect of two different permeable cryoprotectants on freezability of exotic stallion and jack semen. *Indian Journal of Animal Reproduction* **40**(1): 27–30.

Watson P. 1975. Use of a Giemsa stain to detect changes in acrosomes of frozen ram spermatozoa. *Veterinary Record* **97**(1): 12–15.

Yanagimachi R and Bhattacharyya A. 1988. Acrosome-reacted guinea pig spermatozoa become fusion competent in the presence of extracellular potassium ions. *Journal of Experimental Zoology* **248**(3): 354–60.