DUAL STAINING IDENTIFIES A GREATER PROPORTION OF MORIBUND SPERMATOZOA IN STALLION SEMEN WITH POOR CRYOTOLERANCE

NILENDU PAUL[1], THIRUMALA RAO TALLURI[1*], YASHPAL[2], R A LEGHA[2], PRADEEP NAG[1], ARUMUGAM KUMARESAN[1*]

[1]Theriogenology Laboratory, Southern Regional Station of ICAR-National Dairy Research Institute, Bengaluru – 560030, Karnataka, India.

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ABSTRACT

The present study was conducted to evaluate the functional membrane integrity of cryopreserved spermatozoa from stallions belonging to two contrasting motility groups (high and low motility groups). A dual staining method comprising of carboxy fluoroscein diacetate (CFDA) and propidium iodide (PI) was used for this purpose. Stallions were classified into either high (post thaw progressive motility e”35%) or low (post thaw progressive motility <25%) motility groups. The proportion of membrane intact spermatozoa was significantly (P<0.05) higher in high motility group. Interestingly, it was observed that the proportion of moribund population was significantly (P<0.05) higher in low motility group as compared to high motility group. It was inferred that higher proportion of moribund spermatozoa might be one of the important contributing factors for poor sperm motility in stallions belonging to low motile group.

Keywords: *Stallion, spermatozoa, motility, membrane integrity, dual staining*

INTRODUCTION

Stallion semen is considered to be one of the costliest liquids on earth. The importance of preserving spermatozoa from an elite stallion has been realized long ago. However, cryopreservation of stallion spermatozoa encountered several hurdles, which predominantly includes reduction in post thaw sperm quality leading to lesser success rates with artificial insemination using cryopreserved semen as compared to fresh or chilled semen (; Kumar et al., 2019; Sichtar et al., 2019; Talluri et al., 2019; Aurich et al., 2020). Stallion spermatozoa inherently contains higher proportion of poly unsaturated fatty acids on their plasma membrane resulting in higher lipid peroxidation, increased cryo-capacitation like changes, loss of essential molecules due to increased membrane permeability and overall reduction in functional attributes (Aurich et al., 2018). Therefore, it is necessary to assess the functional qualities of spermatozoa accurately to ensure that the semen sample is fit for insemination. Traditionally, sperm viability and membrane integrity are assessed using vital stains like eosin-nigrosine. This staining method identifies two groups of spermatozoa viz. live and dead. However, recently it was identified that a third population called as “moribund” spermatozoa exists in a given ejaculate. The moribund sperm subpopulation represents less viable or dying sperm in a semen sample (Morrel et al., 2010). The importance of moribund spermatozoa has been realized lately as it has been found that they along with dead spermatozoa act as a potential source of reactive oxygen species (ROS), which acts on the viable spermatozoa and significantly affects their functionality including irreversible damage to DNA, lipid peroxidation and loss of motility (Aitken, 2020). With the advent of fluorescent dyes, now it has become possible to identify the moribund spermatozoa subpopulation in a given semen sample.

It is hypothesized that stallions with different levels of sperm cryotolerance might have a different subsets of sperm population. Therefore, a combination of two fluorescent stains carboxyfluoroscein diacetate (CFDA) and propidium iodide (PI) were used to identify the sperm subpopulations based on their functional membrane integrity in high and low motility group stallion semen.

MATERIALS AND METHODS

Cryopreserved stallion semen samples procured from Equine Production Campus, ICAR-NRC on Equines, Bikaner, Rajasthan were utilized for the study. The experiment was conducted at Theriogenology Laboratory of Southern Regional Station of ICAR-NDRI, Bengaluru. Stallions were classified into high motile group (n=3; and post thaw progressive motility e”35%) and low motile group (n=3; and post thaw progressive motility d”25%)

Corresponding Authors: raotalluri79@gmail.com and ogkumaresan@gmail.com

[2]ICAR-National Research Centre on Equines, Hisar, Haryana

based on post-thaw sperm motility. Straws from all the six stallions were thawed at 37°C for 30 seconds and spermatozoa were suspended in sperm TALP (3.1 mMKCl, 100 mMNaCl, 0.29 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2.0 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 550g for 3 min to remove the diluent.

Sperm viability was assessed using a combination of carboxyfluorescein diacetate and propidium iodide (CFDA-PI; Sigma Aldrich, Germany) as described by Paul et al., 2021 Briefly, in this procedure, 10 X 10^6 spermatozoa were taken in sperm TALP and incubated with 5 µl of CFDA (0.5 mg/ml) for 15 minutes at 37°C. After incubation, 2 µl of PI (2.4 mM) was added to the sperm suspension and further incubated for 2 minutes. Then, the sperm suspension was centrifuged, supernatant was removed and a thin smear was made out of the pellet in a clean, grease free 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 1000X magnification. Images from both the filters were later merged to obtain the final image. A minimum of 100 spermatozoa/ per slide were counted and different subpopulations namely live, moribund and dead cells were also evaluated.

Unpaired t test was done to assess the differences in sperm plasma membrane integrity between high and low motile groups. The difference was considered significant when $P < 0.05$. Statistical analysis was performed using GraphPad Prism version 8.4.3.

RESULTS AND DISCUSSION

Using the dual staining method, three different sperm populations were discerned; Spermatozoa with functionally intact membrane (green fluorescence), moribund spermatozoa (greenish yellow fluorescence) and dead spermatozoa (red fluorescence). The three different sperm populations are shown in Fig. 1. The proportion of membrane intact spermatozoa was significantly higher in stallions belonging to high motile group as compared to low motile group. An interesting finding of the study was that the proportion of moribund spermatozoa was significantly higher in stallions belonging to low motile group (45.35 ± 4.12) as compared to high-motile group (26.00 ± 5.52) (Fig. 2).

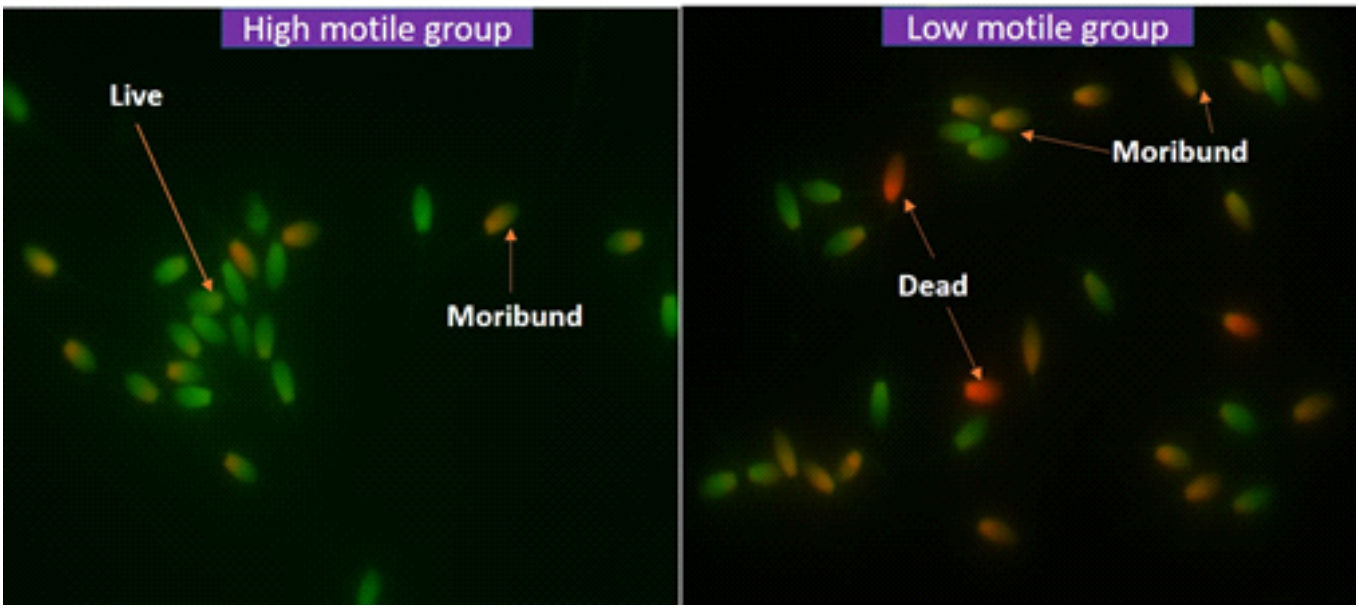

Figure 1- Assessment of stallion sperm membrane integrity using CFDA-PI staining method.

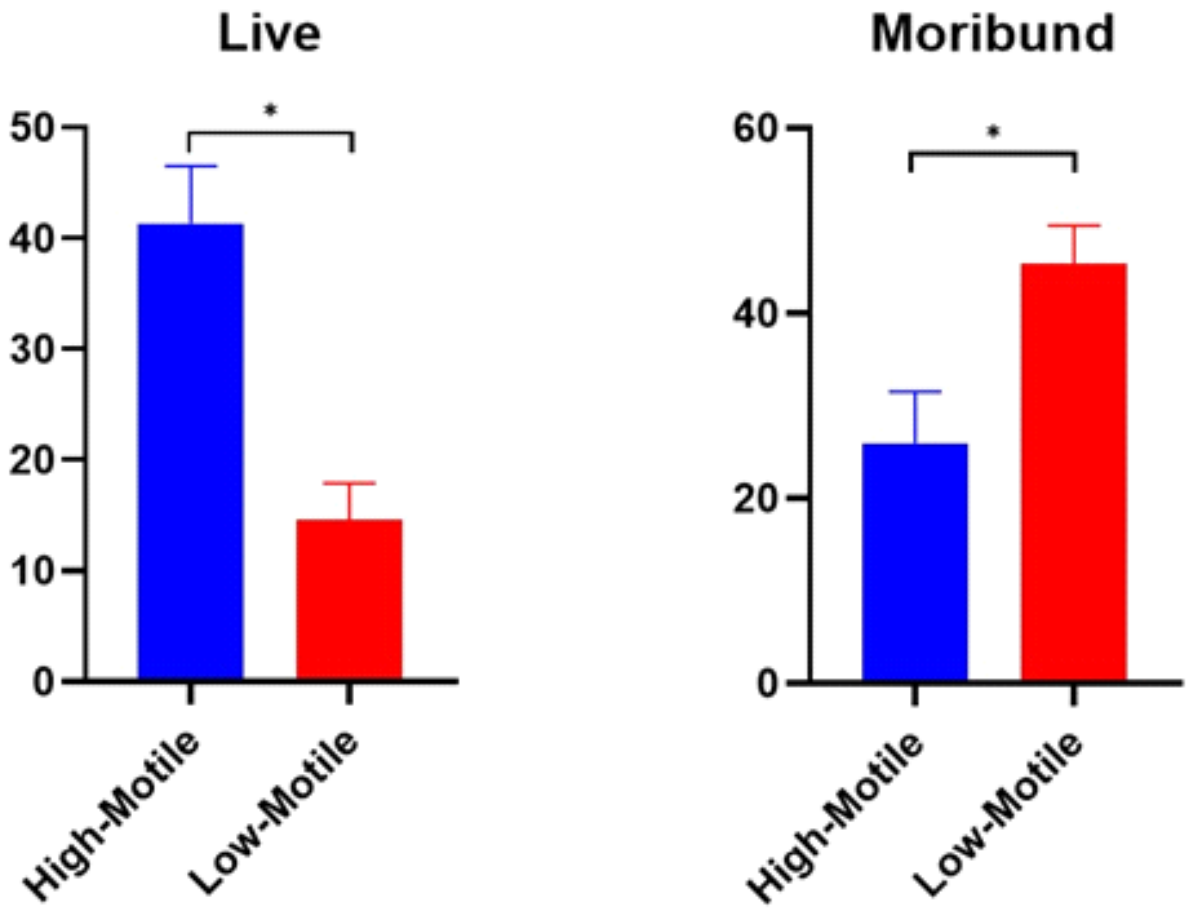

Figure 2- The proportion of live and moribund sperm population in cryopreserved semen of high and low motile group stallions

To achieve success in insemination with frozen semen, spermatozoa must possess certain criteria in terms of their morphology and functionality. Therefore, it is necessary to evaluate the quality of spermatozoa before using for insemination. Spermatozoa must possess certain phenotypic and functional attributes to be able to reach the site of fertilization, establishment of sperm oviduct reservoir and to fertilize the oocyte. It has been reported that spermatozoa with intact plasma membrane, good motility and high mitochondrial membrane potential are preferred for establishment of sperm reservoir at the oviduct for fertilizing the oocyte (Saraf et al., 2017). Intact plasma membrane is an essential pre-requisite for the spermatozoa to take part in fertilization. In this line, several studies have been carried out across many species to assess sperm membrane integrity using advanced fluorescent based staining techniques (Dolník et al., 2019).

In the present study, we used CFDA and PI for assessment of functional status of stallion spermatozoa. CFDA is a membrane-permeant colourless substrate that is rapidly converted into a membrane-impermeant green fluorescent derivative by intracellular esterases in metabolically active cells (Colenbrander et al., 2003). PI is membrane-impermeant red fluorescent molecule that enter the nucleus of a cell in which the plasma membrane is damaged. When used in combination, cells with damaged membranes fluoresce red as PI enters the cell, and as intracellular esterases leaked from the cell, CFDA could not be converted into its green derivative. Using the dual fluorescent staining method, it was observed that higher proportion of membrane compromised spermatozoa were present in low motile group as compared to the high motile group. Previous studies conducted on other species also found that low quality semen sample had higher proportion of moribund spermatozoa, which is in line with our present finding (Singh et al., 2016; Paul et al., 2021). Interestingly, the present study recorded a higher proportion of moribund spermatozoa in stallions of to low motility group. Moribund spermatozoa are characterized by presence of compromised plasma membrane, which undergo transition from a living to a moribund state, and ultimately die (Shojaei et al., 2012). The released ROS from the moribund sperm population affects the functionality of

the normal contemporary spermatozoa and reduced their functionality. Thus, identification of the proportion of moribund spermatozoa is of significance.

CONCLUSION

It was inferred that higher proportion of moribund spermatozoa might be one of the important contributing factors for poor sperm motility in stallions belonging to low motile group. Since moribund sperm population cannot be assessed by routine semen evaluation, it is suggested that use of the dual fluorescent staining method would provide valuable information to the stallion sperm functional assessment.

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