*Indian Journal of Animal Sciences* **95** (4): 315–321, April 2025/Article
https://doi.org/10.56093/ijans.v95i4.166560

# Simultaneous detection of equine sperm subpopulations with different phenomes using a novel staining technique

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Received: 13 May 2025; Accepted: 30 June 2025

ABSTRACT

Nowadays, there is an increased demand for liquid and frozen semen of stallion as the breeding registries have allowed artificial inseminations. Therefore, it is important to ensure the quality of stallion semen because it will be used for inseminating a large number of mares. Traditionally, various dyes were being used for assessing individual sperm functional parameters which is laborious, time consuming and require large quantity of samples. Recently, fluorescent dye-based sperm quality assessment techniques are replacing the conventional staining procedures and are proving to be more accurate, reliable and time efficient. In the current study, we developed fluorescent dye based triple staining methods for simultaneous detection of sperm vital parameters i.e viability, acrosome integrity, mitochondrial membrane potential and mitochondrial reactive oxygen species. Cryopreserved semen from 6 stallions were used for the current study. Through the standardized triple staining methods, we identified various sperm subpopulations (seven sub populations in FITC-PNA+MitoSox+Hoechst 33258 and four subpopulations in FITC-PNA+JC-1+Hoechst 33258), all of which cannot be assessed using single or dual staining techniques. We therefore conclude, that triple staining can be useful for simultaneous assessment of important sperm functional parameters of stallion spermatozoa in a cost and time effective manner.

**Keywords**: Fluorescent microscopy, Mitochondria, Reactive oxygen species, Spermatozoa, Stallion, Subpopulation

Assessment of sperm functional parameters is of great value to identify and select elite males for breeding purpose. The number of tests and assays available to assess sperm quality has increased dramatically in the last decade. Currently, several tests are available for screening and assessing the sperm quality and functionality. However, not all the tests available are true reflectors of sperm fertilizing potential. Development of sperm technologies and improvement in the diagnosis of andrological conditions require improvements of sperm diagnostic techniques together with a better understanding of the molecular basis of spermatozoa dysfunction (Peña *et al*. 2016). The field of animal andrology has witnessed a rapid shift in sperm quality evaluation starting from conventional dyes to more advanced and accurate fluorophore-based dyes. With the aid of fluorescent based dyes, sperm assessment became easy, more accurate, reliable and saved the time required for sample processing and analysis.

Fluorescent dyes are commonly used for assessing sperm phenotypic characteristics including plasma membrane integrity, acrosome intactness, mitochondrial membrane potential, plasma membrane lipid peroxidation status, intracellular calcium level, apoptosis (Singh *et al*. 2016, Boe-Hansen and Satake 2019, Bucher *et al*. 2019, Ugur *et al*. 2019, Palacín *et al*. 2020, Paul *et al*. 2020, Gallo *et al*. 2021, Nag *et al*. 2021). These tests have been shown to be effective and reliable for identifying the aforementioned parameters, when the test was carried out either individually (single staining) or combined with another dye (dual staining). This results in need of more samples for analysis as well as higher cost involvement due to more usage of dyes. Hence, modification of existing fluorescent staining techniques may result in better utilization of resources without compromising the efficiency of sperm quality assessment. Several studies report use of fluorescent dyes for sperm quality evaluation across species. Out of these, some tests are strongly related to male fertilizing potential whereas other tests were found to be a weak predictor of fertility (Singh *et al*. 2016, Kumaresan *et al*. 2017). Therefore, it is logical to perform a handful of tests that have close and tight association with spermatozoa fertilizing potential rather than performing all the tests available. Selecting the right combination of tests thus can be helpful in rapid sperm quality assessment which will be economical and more suitable for large amount of sample evaluation. Nowadays, triple stain combination is

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emerging as a tool to assess different attributes of sperm contemporaneously without spending much time separately for assessing each individual attribute. Triple staining technique has been used in sperm quality assessment in animals such as ram, boar and bovines (Nagy *et al*. 2003, Torres *et al*. 2016, Santos *et al*. 2018). One of the crucial factors in triple staining technique is selecting the right combination of dyes for simultaneous assessment of different vital functional parameters of given ejaculate.

Stallion spermatozoa differ from other livestock species in terms of molecular composition, vulnerability to cold shock, longevity and susceptibility to reactive oxygen species (Baumber *et al*. 2003, Colenbrander *et al*. 2003, Guthrie and Welch 2012, Delgado-Bermúdez *et al*. 2019, Medica *et al*. 2021). Therefore, traditional semen analysis might not hold good for understanding the critical factors important for sperm quality. Moreover, increased use of cooled and frozen semen in the equine industry warrants the need for reliable tests to determine sperm quality. The ability to accurately determine the quality of sperm will enable the equine practitioner to better predict the expected fertility of stallion semen after cryopreservation or cooling. Cryopreserved stallion spermatozoa exhibit cryocapacitation like changes resulting in pre-mature acrosome reaction leading to reduced fertility. moreover, during the process of cryopreservation, the functionality of Mitochondria is also altered (Ezzati *et al*. 2020). Maintaining mitochondrial functionality is important in stallion spermatozoa due to a higher dependence of oxidative phosphorylation in this species (Davila *et al*. 2016). Moreover, due to increased content of polyunsaturated fatty acids in stallion sperm membrane, they are prone for oxidative damage. Supraphysiological ROS production in stallion spermatozoa has been shown to compromise mitochondrial function thereby affecting sperm motility (Del Prete *et al*. 2019). In other species, a close relationship between high levels of intracellular ROS and male reproductive dysfunctions have been demonstrated promoting the development of different methods of ROS detection and quantification (Gallo *et al*. 2021). Therefore, the importance of assessing sperm viability, membrane integrity, acrosomal integrity, mitochondrial membrane potential and reactive oxygen species in stallion is well recognized (Peña *et al*. 2016, Peña *et al*. 2018, Hernández-Avilés *et al*. 2021). Keeping in view the importance of the above cited sperm functional parameters, in the current study, two triple staining combinations were developed for simultaneous assessment of plasma membrane integrity, acrosome reaction status, mitochondrial membrane potential and mitochondrial reactive oxygen species generation in stallion spermatozoa. It is hoped that these triple-staining assays have a potential practical value in assessing stallion sperm quality and cryobiological research because of their better efficiency, reliability and less time consuming as compared to already available conventional or single or dual staining techniques.

## MATERIALS AND METHODS

The current study was conducted at the Theriogenology laboratory, Southern Regional Station of ICAR- National Dairy Research Institute, Bengaluru, Karnataka, India. Prior approval of the Institutional Animal Ethics Committee was obtained for all the experimental procedures and performed in accordance with relevant guidelines and regulations (CPSCEA/2019/NRCE/EPC-IXX14589).

*Source of sample and semen cryopreservation*: Semen samples used in the present experiment were collected from six adult stallions (age ranging from 4 to 6 years) maintained at Equine Production Campus, ICAR-National Research Centre on Equines, Bikaner, Rajasthan, India. Semen collection was performed early in the morning hours using an oestrus mare as dummy. Semen was collected from stallions using a pre-warmed (45-50ºC) Colorado model artificial vagina lubricated with liquid paraffin and fitted with an inline filter to separate the gel fraction. In order to reduce the extra-gonadal sperm reserves, semen was collected over a period of four consecutive days before the commencement of the actual experiments. The stallions were given two days of sexual rest before the beginning of the experiment. After that, two ejaculates were collected from each stallion. Immediately after collection, ejaculates were transported to the laboratory and kept at 37ºC for until processing. The semen samples were centrifuged at 650 g for 5 minutes to obtain the sperm pellet, and the supernatant was discarded. The sperm pellet was further extended in a secondary freezing extender (6.0 g glucose, 11.00 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 the 5% of dimethyl formamide (DMF) as cryoprotectant. The extended semen was equilibrated for 2 hours at 4ºC in a cooling cabinet and, later the extended semen was filled in 0.5 ml French medium straws and sealed with an automatic filing and sealing machine. The semen straws were frozen using programmed freezer. Cryopreserved stallion semen straws were thawed at 37ºC for 30 seconds and the sperm was suspended in Tyrode's albumin lactate pyruvate (sperm TALP) (having 3.1 mM KCl, 100 mM NaCl, 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 to remove the extender and egg yolk particles by centrifugation at 300 g for 3 min before performing the experiments.

*Triple staining of spermatozoa*: For assessing the various sperm functions at a time, we have devised two different combinations of triple staining methods, which are explained below. Fluorescent dyes such as Hoechst 33258, MITOSOX and JC-1 procured from InvitrogenTM, Thermo Fisher scientificTM, USA and FITC-PNA was procured from Sigma-AldrichTM, USA. The flow of steps involved in these staining procedures of triple staining technique are shown in fig. 1 and 2 and explained below.

*Simultaneous assessment of plasma membrane integrity,*

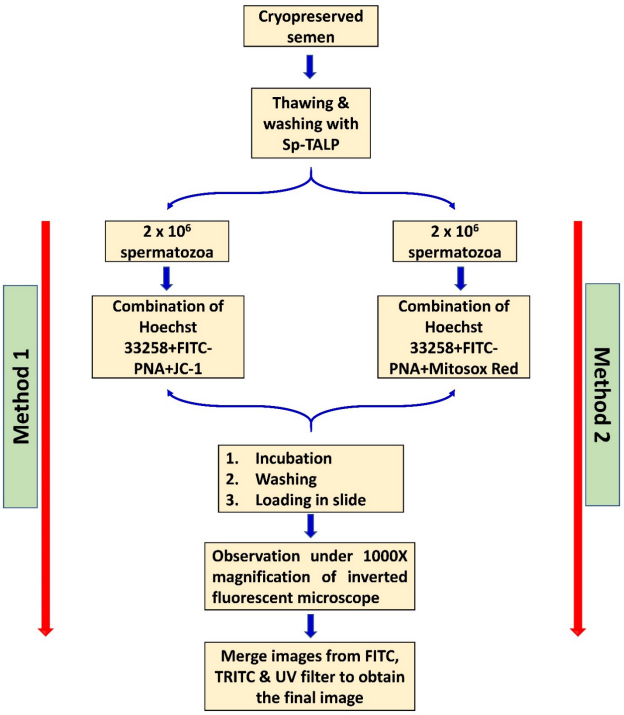


Fig. 1. Flow chart indicating triple staining protocol using two different combinations

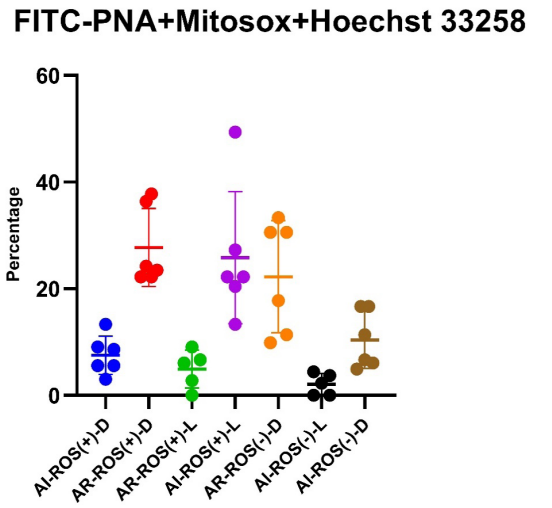


Fig. 2. Proportion of different sperm subpopulations identified using a combination of FITC-PNA, MitoSox and Hoechst 33258 staining. AI: Acrosome intact, AR: Acrosome reacted; ROS(+): High mitochondrial reactive oxygen species, ROS(-): Low mitochondrial reactive oxygen species, L: Live, D: Dead

*acrosome intactness and mitochondrial superoxide*: In this technique, we used a combination of three fluorescent dyes i.e. Hoechst 33258, FITC-PNA and Mitosox Red for simultaneous assessment of viability, acrosome reaction status and mitochondrial superoxide generation. Briefly, washed spermatozoa (2 million) were taken in 1.5 mL tube, incubated with working solutions of Hoechst 33258 (9 µL, 40µM working solution), FITC-PNA (1µL,1mg/mL) and MITOSOX (1 µL, 500 µM) for 15 min. After incubation, 200 µL of sperm TALP was added and washed. The supernatant was removed and a drop of sperm pellet was smeared on a clean grease free pre- warmed slide and upon air drying 10 µL DABCO (anti-fading agent) was added. The smear was covered with a coverslip and observed under fluorescent microscope (Nikon ECLIPSE Ti-s, Japan) using FITC, TRITC and UV filter under high magnification (1000X). The images from all three filters were later merged to obtain the final image.

*Simultaneous assessment of plasma membrane integrity, acrosome intactness and mitochondrial membrane potential*: Sperm preparation was similar to the procedure as explained above. In this method, Mitosox Red was replaced with with JC-1 (5,5,6,6'-tetrachloro-1,1',3,3' tetraethylbenzimi-dazoylcarbocyanine iodide) dye to assess sperm mitochondrial membrane potential along with sperm viability and acrosome reaction status. Briefly $2x10^6$ of washed sperm pellet was stained with working solutions of Hoechst 33258 (9 µL, 40µM working solution), FITC-PNA (1µL,1mg/mL) and JC-1 (6 µL, 0.2 mM), respectively and incubated for a period of 20 minutes. Upon incubation, the excess dye was removed by washing with sperm TALP. A fraction of resultant pellet was then smeared on a microslide and air dried. DABCO was added on the smear and covered with cover slip. The images were obtained and visualized as mentioned earlier.

*Statistical analysis*: All the statistical analysis were performed using GraphPad Prism version 8.4.3. Proportion of different subpopulations were expressed as percentage. Pearson correlation was employed to assess the relationship between different sperm subpopulations and motility. The data was considered significant at $p \leq 0.05$ level.

## RESULTS AND DISCUSSION

Use of a single fluorophore can identify only fewer sperm subpopulation (maximum of 2) in the given semen sample (Table 1). However, the combination of three dyes for simultaneous detection of various sperm subpopulations could identify eight different kinds of subpopulations. Possible combinations of sperm subpopulations that could be detected using triple staining are given in Table 2. Out of all the possible combinations, the detected sperm subpopulations are presented in the Table 3 and 4. The

Table 1. Sperm subpopulations identified using single staining method

| Single staining technique | Sperm subpopulation |
|---|---|
| FITC-PNA | Acrosome intact |
| | Acrosome reacted |
| JC-1 | High MMP |
| | Low MMP |
| Mitosox | ROS (+) |
| | ROS (-) |
| Hoechst 33258 | Viable |
| | Dead |

Table 2. Possible theoretical sperm subpopulations identified using triple staining method

| Triple staining combination | Possible sperm subpopulations |
|---|---|
| FITC-PNA+ Mitosox+ Hoechst 33258 | Acrosome intact, ROS (-) and viable |
| | Acrosome intact, ROS (-) and dead |
| | Acrosome intact, ROS (+) and viable |
| | Acrosome intact, ROS (+) and dead |
| | Acrosome reacted, ROS (-) and viable |
| | Acrosome reacted, ROS (-) and dead |
| | Acrosome reacted, ROS (+) and viable |
| | Acrosome reacted, ROS (+) and dead |
| FITC-PNA+ JC-1+ Hoechst 33258 | Acrosome intact, high MMP and viable |
| | Acrosome intact, high MMP and dead |
| | Acrosome intact, low MMP and viable |
| | Acrosome intact, low MMP and dead |
| | Acrosome reacted, high MMP and viable |
| | Acrosome reacted, high MMP and dead |
| | Acrosome reacted, low MMP and viable |
| | Acrosome reacted, low MMP and dead |

proportion of different sperm subpopulations identified using two different combinations of triple staining namely FITC-PNA+ Mitosox+ Hoechst 33258 and FITC-PNA+JC-1+ Hoechst 33258 are shown in Fig. 3 and 4. By using the first triple staining combination (FITC-PNA+ Mitosox+ Hoechst 33258), we recorded six subpopulations, while using second triple staining combination (FITC-PNA+JC-1+ Hoechst 33258), we observed four subpopulations of spermatozoa.

Using the first triple stain combination, the subpopulations were categorized as acrosome intact (AI) or acrosome reacted (AR), reactive oxygen species

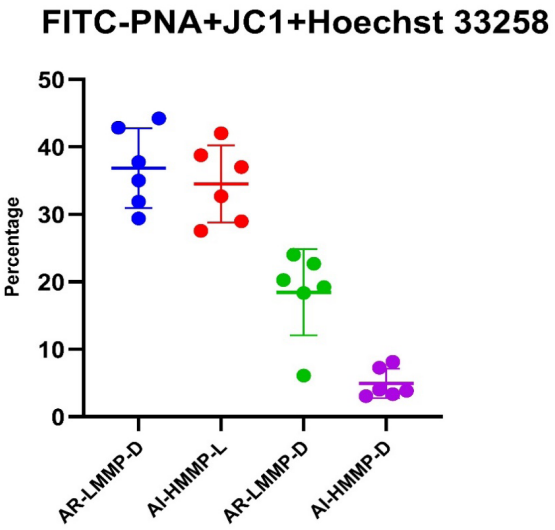


Fig. 3. Proportion of different sperm subpopulations identified using a combination of FITC-PNA, JC-1 and Hoechst 33258 staining. AI: Acrosome intact, AR: Acrosome reacted, HMMP: High mitochondrial membrane potential, LMMP: Low mitochondrial membrane potential, L: Live, D: Dead

Table 3. Identified sperm subpopulations using FITC-PNA+ Mitosox red+ Hoechst 33258 triple stain

| Stallion sperm subpopulation | FITC-PNA | Mitosox-Red | Hoechst 33258 |
|---|---|---|---|
| Acrosome intact, ROS (+) and dead | - | + | + |
| Acrosome reacted, ROS (+) and dead | + | + | + |
| Acrosome reacted, ROS (+) and viable | + | + | - |
| Acrosome intact, ROS (+) and viable | - | + | - |
| Acrosome reacted, ROS (-) and dead | + | - | + |
| Acrosome intact, ROS (-) and viable | - | - | - |

Table 4. Identified sperm subpopulation using FITC-PNA+ JC-1+ Hoechst 33258 triple stain

| Stallion sperm subpopulation | FITC-PNA | JC-1 | Hoechst 33258 |
|---|---|---|---|
| Acrosome reacted, low MMP and viable | + | Green | - |
| Acrosome intact, high MMP and viable | - | Red-orange | - |
| Acrosome intact, low MMP and dead | - | Green | + |
| Acrosome intact, high MMP and dead | - | Red-orange | + |

(ROS) positive or negative and viable (L) or dead (D). In the similar fashion, using the second triple staining method we categorized the subpopulations as acrosome intact (AI) or acrosome reacted (AR), high mitochondrial membrane potential (HMMP) or low mitochondrial membrane potential (LMMP) and viable (L) or dead (D). Representative pictures of triple stained spermatozoa using

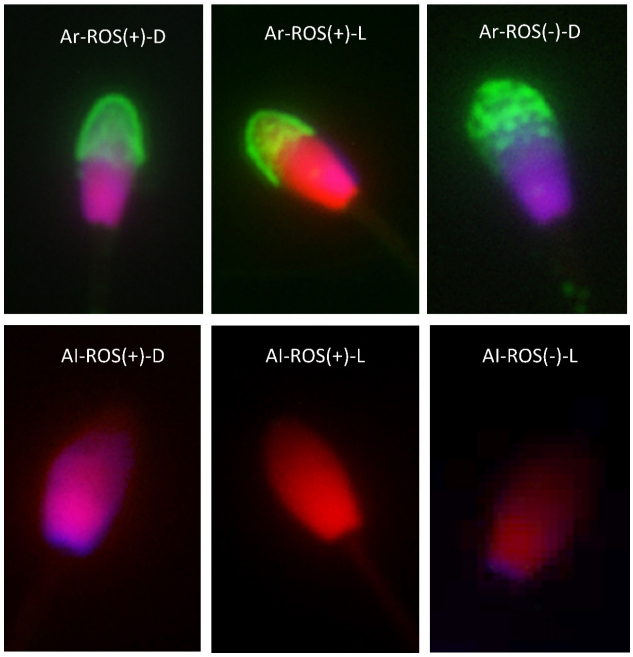


Fig. 4. Representative pictures of different sperm subpopulations identified using a combination of FITC-PNA, MitoSox and Hoechst 33258 staining. AI: Acrosome intact, Ar: Acrosome reacted, ROS(+): High mitochondrial reactive oxygen species, ROS(-): Low mitochondrial reactive oxygen species, L: Live, D: Dead

both the methods are depicted in Fig. 5.

Among the different sperm subpopulations identified in the first combination of triple staining, the proportion of acrosome reacted-mitosox positive-dead, acrosome intact-mitosox positive-live and acrosome reacted-mitosox negative-dead spermatozoa were higher. In contrast, acrosome intact-mitosox negative-dead, acrosome reacted-mitosox positive-live and acrosome intact-mitosox negative-live were lower in proportion in all of the samples analyzed. Among the different subpopulations identified in the second combination of triple staining, the proportion of acrosome reacted- low MMP- dead spermatozoa and acrosome intact-high MMP-live spermatozoa were higher. Alternatively, acrosome intact- low MMP- dead and acrosome intact- high MMP- dead spermatozoa were lower in proportion.

Spermatozoa, while traversing through female reproductive tract encounters a series of barriers to reach the site of fertilization in oviduct (Suarez and Pacey 2006). The spermatozoa population reaching to oviduct is significantly decreased during the filtration process and removal of abnormal spermatozoa by maternal immune system. Thereafter, the spermatozoa form a reservoir in oviduct and waits for the arrival of the ova. For establishing sperm-oviduct reservoir and to fertilize an oocyte, spermatozoa must possess certain functional attributes which are not only restricted to motility (Saraf *et al*. 2019). Thus, assessment of sperm functionality using specific fluorophores is important for ensuring high fertility. Fluorescent probes have been used in bull, ram, boar, and stallion spermatozoa to evaluate the integrity of the sperm membrane, acrosome, and mitochondrial membrane potential (MMP) of sperm and provides better interpretation in comparison to conventional techniques. The semen consists of heterogenous subpopulations of spermatozoa, and each differs in their fertilizing potential (Quintero-Moreno *et al*. 2003, Celeghini *et al*. 2010, Ramón *et al*. 2014). Therefore, identifying the maximum number of subpopulations simultaneously using triple staining reflects the overall quality of semen sample assumes higher importance.

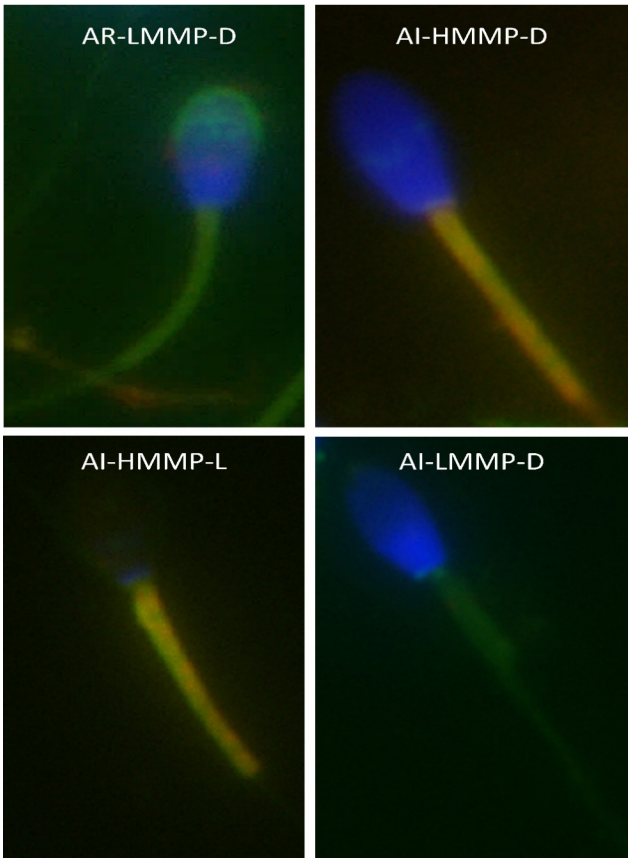


Fig. 5. Representative pictures of different sperm subpopulations identified using a combination of FITC-PNA, JC-1 and Hoechst 33258 staining AI: Acrosome intact, AR: Acrosome reacted, HMMP: High mitochondrial membrane potential, LMMP: Low mitochondrial membrane potential, L: Live, D: Dead

In the present study, both the triple staining techniques proved to be effective in identifying several subpopulations in a given semen sample. We have developed two kinds of triple staining techniques and showed that this protocol is effective in identifying more subpopulations as compared to individual fluorescent based staining techniques. The vital sperm functional parameters such as viability, acrosome status, mitochondrial membrane potential as well as mitochondrial superoxide generation can be rapidly detected using the technique which reduces the time for individual sample processing prior to use of single or dual stain. Earlier report on triple staining technique for simultaneous assessment of plasma membrane integrity, acrosome integrity and mitochondrial membrane potential of ram spermatozoa using PI, FITC-PSA and JC1 has found it to be efficient in evaluating the aforementioned parameters (Celeghini *et al*. 2010). Similarly, simultaneous assessment of bull sperm functional parameters was performed using a combination of SYBR-14, PI, PE–PNA (phycoerythrin-conjugated peanut agglutinin), and MTDR (MitoTracker Deep Red) which was found to be accurate in identifying various subpopulations important for sperm functionality and fertilizing ability (Kanno *et al*. 2016).

The acrosome reacted-mitosox positive-dead sperm and acrosome reacted- low MMP- dead sperm were the subpopulations in highest proportions in the first and second combination of triple staining, respectively. As the proportion of mitosox stained spermatozoa were reported to be inversely correlated with MMP in previous study (Chai *et al*. 2017, Gallo *et al*. 2021), both methods used in our study are revealing the same kind of subpopulation. Using first combination of triple staining technique, we detected that a higher proportion of spermatozoa were dead with reacted spermatozoa and mitosox +ve i.e., higher mitochondrial superoxide generation. This is in line with the fact that dead spermatozoa are a potent source for ROS generation. Thus, this subpopulation may hamper the functionality of spermatozoa. Interestingly, we also found

a certain proportion of live sperm subpopulation with intact acrosome but possesses higher superoxide content. There is a possibility that this subpopulation is representative of moribund spermatozoa which may also acts as a potent source for ROS generation leading to reduction in quality of the normal spermatozoa (Sutovsky 2015). Besides their basic role in ATP synthesis, mitochondria give rise to reactive oxygen species (ROS) as by-products of their activity. ROS is a large class of molecules that includes radicals (hydroxyl ion, superoxide, nitric oxide, peroxyl, etc.), non-radicals (ozone, single oxygen, lipid peroxides, hydrogen peroxide), and oxygen derivatives.

Though, the sperm mitochondrial functionality was measured previously by different fluorophores, such as Rhodamine 123, or JC-1, the mitochondrial membrane potential was also evaluated, which is highly correlated with sperm motility. MMP closely reflects mitochondrial function, so its assessment allows predicting sperm mitochondrial function and performing a complete evaluation of sperm quality. Indeed, this parameter has been demonstrated to be associated with functional sperm parameters. Using the second combination of triple staining, we could detect that a higher proportion of acrosome reacted spermatozoa were dead exhibiting low MMP. On the other hand, a higher proportion of spermatozoa with intact acrosome were live and retained their MMP. This subpopulation possesses higher potential to establish sperm reservoir in female reproductive tract and to successful accomplishment of fertilization. Thus, an ejaculate can be selected prior to storage or insemination if it contains higher proportion of spermatozoa having membrane intactness, non-reacted acrosome and high MMP with low ROS levels. The assays we used in this study, validated that triple stain technique is an efficacious method for the simultaneous assessment of sperm vitality, MMP and acrosome status to find out the fertilizing capacity of the given sample.

Two combinations of fluorescent dye based triple staining techniques have been devised in this study, which have a greater accuracy of identifying various sperm subpopulations that directly or indirectly influence the overall sperm quality and fertilizing potential. Thus, triple staining technique can be employed for stallion sperm quality assessment in identifying quality sperm producing stallions and to disseminate the superior germplasm for obtaining better conception rates when used for AI in mares.

## ACKNOWLEDGEMENTS

Authors are thankful to the Director, ICAR-National Dairy Research Institute, India for providing necessary facilities for carrying out this research. The authors also express their gratitude to the ICAR-National Research Centre on Equines, Hisar, Haryana for providing stallion semen.

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