Comparison of Two Methods for Sperm Plasma Membrane Integrity Assessment in Frozen Murrah Buffalo Semen

Rahayu JD[1], Purwantara B[2], Said S[3], Arifiantini RI[2*]

[1] Reproductive Biology Study Program, Post Graduate School, IPB University
Jl. Agatis Kampus IPB Darmaga, Bogor 16680, Indonesia
[2] Division of Reproduction and Obstetrics, School of Veterinary Medicine and Biomedical Sciences, IPB University
Jl. Agatis Kampus IPB Darmaga, Bogor 16680, Indonesia
3Research Centre for Applied Zoology, National Research and Innovation Agency
Jl. Raya Jakarta-Bogor No.Km. 46, Cibinong, Bogor 16911, Indonesia
**E-mail: Arifiantini@apps.ipb.ac.id*

(received 06-01-2025; revised 16-03-2025; accepted 17-03-2025)

ABSTRAK

Rahayu JD, Purwantara B, Said S, Arifiantini RI. 2025. Perbandingan dua metode untuk penilaian integritas membran plasma sperma dalam semen beku kerbau murrah. JITV 30(1):19-27. DOI:http://dx.doi.org/jitv.v30i1.3488.

Integritas membran plasma sperma dalam semen beku sangat penting dalam menilai kualitas semen dan secara langsung terkait dengan kesuburan. Integritas membran plasma sperma dapat diuji menggunakan beberapa metode, termasuk Uji Hypo-Osmotik Swelling (HOS) Tes dan pewarnaan Carboxyfluorescein Diacetate-Propidium Iodide Staining (CFDA-PI). Penelitian ini membandingkan integritas membran plasma semen kerbau Murrah beku menggunakan HOS-Tes dan CFDA-PI. Semen diperoleh dari empat kerbau jantan, masing-masing mewakili kelompok produksi yang berbeda. Semen beku dicairkan secara, dan kualitasnya dinilai. Integritas membran plasma setiap sampel dievaluasi menggunakan metode pewarnaan Uji HOS dan CFDA-PI. Uji-T dua sampel digunakan untuk menganalisis data dan menunjukkan bahwa terdapat perbedaan yang signifikan secara statistik dalam integritas membran plasma sperma antara metode HOS-Tes (64,83%) dan pewarnaan CFDA-PI (58,97%) (P<0,05). Penilaian HOS-Tes optimal untuk efektivitas biaya, kecepatan, dan kenyamanan. Uji ini dapat diterapkan di laboratorium dan lapangan dengan kemudahan yang sama. Pewarnaan CFDA-PI sebaliknya, merupakan standar yang baik untuk akurasi dan presisi. Hal ini memberikan dasar yang komprehensif untuk menentukan kualitas semen beku.

Kata Kunci: CFDA-PI, HOS-tes, Kerbau Murrah, Membran Plasma Sperma

ABSTRACT

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The integrity of the sperm plasma membrane in frozen semen is crucial in assessing semen quality and is directly linked to fertility. The plasma membrane integrity of sperm can be tested using several methods, including the Hypo-Osmotic Swelling Test (HOS-Test) and Carboxyfluorescein Diacetate-Propidium Iodide Staining (CFDA-PI). This study compares the integrity of the plasma membrane of frozen Murrah buffalo semen using the HOS-Test and CFDA-PI. The semen was obtained from four buffalo bulls, each representing a different production batch. The straw was thawed individually, and quality was assessed. The plasma membrane integrity of each sample was evaluated using the HOS-Test and CFDA-PI staining methods. A two-sample t-test was used to analyze the data and demonstrate that there is a statistically significant difference in sperm plasma membrane integrity between the HOS-Test (64.83%) and CFDA-PI staining (58.97%) methods (P<0.05). The HOS-Test assessment is optimal for cost-effectiveness, speed, and convenience. It can be implemented in laboratory and field settings with equal ease. In contrast, CFDA-PI staining is a good standard for accuracy and precision. It provides a comprehensive foundation for determining the quality of frozen semen.

Key Words: CFDA-PI, HOS-Test, Murrah Buffalo, Sperm Plasma Membrane

INTRODUCTION

The buffalo (*Bubalus bubalis*) is one of the large ruminants with considerable potential for development in Indonesia. One of the breeds of buffalo found in Indonesia is the Murrah buffalo. The Murrah buffalo is a dairy breed that produces meat and livestock (Rajoriya et al. 2016). Buffalo breeding can be conducted through either natural mating or artificial insemination. The utilization of artificial insemination in buffaloes has the potential to enhance the genetic quality of the progeny, as it involves the use of frozen semen from selected superior bulls. In artificial insemination programs, frozen semen plays an important role in improving the genetic quality of the sperm. After freezing, a frozen semen evaluation must be carried out to ensure that the

semen used for insemination is of good quality. The frozen semen used in the AI program must meet the minimum post-thaw motility (PTM) standard of 40% (SNI 2021). In addition, the semen must have optimal sperm motility, viability, intact plasma membrane and acrosome integrity, normal morphology, and high deoxyribonucleic acid (DNA) content to facilitate oocyte fertilization (Chelucci et al. 2015).

All parts of the sperm are covered by a plasma membrane, which acts as a protection against environmental changes and as a transport system for components from the inside of the cell to the outside or vice versa, maintaining the integrity of the biochemistry and structure of the sperm (Trimble & Grinstein 2015). Hence, the integrity of the plasma membrane determines sperm quality. Sperm with good plasma membrane integrity can survive, undergo complex changes in the female reproductive organs, and fertilize eggs. Damage to the plasma membrane and acrosome reduces the fertility potential of sperm (Morrell et al. 2018). Therefore, assessing active and functional plasma membrane integrity is essential for successful fertilization.

The assessment of sperm plasma membrane integrity can be performed on both fresh and frozen sperm. The most commonly employed methodology for evaluating the integrity of the sperm plasma membrane is the hypo-osmotic swelling test (HOS-Test). The fundamental premise of this methodology is the capacity of the sperm plasma membrane to undergo osmotic expansion within a hypo-osmotic milieu. The capacity to identify sperm damage is advancing with technological and scientific advancement (Moore & Hasler 2017). Fluorescent agents were initially used to assess mammalian sperm plasma membrane integrity, as delineated by Harrison & Vickers (1990). One of the dyes employed is carboxyfluorescein diacetate and propidium iodide (CFDA-PI). CFDA-PI dye is widely used in sperm quality assessment to evaluate sperm viability and membrane integrity. This dye is very effective in identifying live and dead sperm. It provides information on the integrity of the sperm membrane, which is very important in determining the ability of the sperm to fertilize an egg.

The study of Murrah buffalo plasma membrane integrity using HOS-Test and CFDA-PI staining has yet to be previously reported in the scientific literature. The HOS test can provide a good assessment of the ability of the sperm plasma membrane to function physiologically. In contrast, CFDA-PI staining provides a more comprehensive assessment of sperm viability and membrane integrity, with more quantitative and objective results. Therefore, the objective of this study was to compare the integrity of the plasma membrane of frozen Murrah buffalo sperm using HOS-Test and CFDA-PI staining. The two methods (HOST and CFDA-PI) were chosen to represent the conventional and state-of-the-art methods. The results are expected to provide more accurate integrity testing information for plasma membranes.

MATERIALS AND METHODS

All procedures in this study were approved by The Ethical Committee of the National Research and Innovation Agency (BRIN) under No: 093/KE.02/SK/05/2023.

Research time and place

The study was conducted between November and December 2023 at the Stem Cell Laboratory, located within the Genomics Building of the National Research and Innovation Agency, Bogor. It employed frozen semen samples from the Lembang Artificial Insemination Centre (AIC). The methodology for producing frozen semen adheres to the standards set forth by the Indonesian National Standard SNI 4869-2:2021.

Research procedure

Frozen semen was derived from four buffalo bulls aged between 10 and 12 years, with six batch codes selected at random, each consisting of five straws. Six batch codes were necessary to ensure the data collected from the study could be transmitted accurately and consistently. Consequently, 120 straws were utilized. The frozen semen is initially thawed using a water bath at 37°C for 30 seconds, after which it is transferred to a micro-tube and stored at 37°C for observation.

Sperm motility evaluation

The sperm motility was observed using a computer-assisted sperm analysis (CASA) system. A volume of 5 µl of semen was deposited onto an object glass and covered with a cover glass. The sperm were analyzed in four fields with a cell count of 300–400 cells using the software program Sperm Vision (Minitüb, Tiefenbach, Germany) on a warming table at 37°C.

Sperm viability evaluation

The viability of sperm was evaluated using eosin-nigrosin staining by the methodology proposed by Kumar et al. (2017). In this staining technique, live sperm remain unstained and appear transparent, whereas dead sperm are stained red. The viability of the sperm was determined by enumerating the live and dead cells across a minimum of 10 fields of view, with a minimum of 200 cells evaluated in each sample.

Acrosome status evaluation

The sperm's acrosome status was determined by staining with Fluorescein Isothiocyanate Peanut Agglutinin (FITC-PNA). The staining process was conducted in a dark room to prevent external light sources from affecting the results. A minimum of 200 cells were observed under a fluorescence microscope.

Sperm morphology evaluation

The sperm morphology was observed using the carbolfuchsin-eosin dye (William's stain), following the established methodology. The sperm morphology was observed under a microscope with 400x magnification, and a minimum of 200 normal and abnormal sperm were counted.

Hypo Osmotic Swelling Test (HOS-Test)

The HOS-Test method for assessing sperm membrane integrity is based on the methodology proposed by Agarwal et al. (2016). The HOS solution was maintained at 37°C in a water bath. A 30 µl sperm sample was added to 300 µl of HOS solution, homogenized, and incubated at 37°C. Subsequently, following a 30-minute incubation period, the integrity of the sperm membrane in frozen semen was assessed by adding 5 µl of the solution mixture to a microscope slide, which was then covered with a cover slip and observed under a microscope at 400x magnification. The sperm exhibiting intact plasma membranes exhibited a curved tail reaction. In contrast, a straight tail indicated damage to the plasma membranes. The total number of sperm was at least 500, and the number of reacted and unreacted sperm was enumerated.

Carboxyfluorescein Diacetate-Propidium Iodide (CFDA-PI)

The evaluation of sperm plasma membrane integrity with the CFDA-PI dye (Sigma Aldrich, Germany) is based on the methodology outlined by Harrison & Vickers (1990), with minor modifications. The CFDA dye stock solution was prepared by dissolving the dye in 4 mg/mL dimethyl sulfoxide (DMSO). The PI dye stock solution was prepared by dissolving the dye in 0.5 mg/mL PBS (both solutions were prepared in a dark room and stored at -20°C). A 20 µL aliquot of frozen-thawed buffalo semen was combined with 40 µL of CFDA dye and 5 µL of PI dye and incubated for 10 minutes at 37°C in the dark. Subsequently, 5 µL of the sample was transferred to a microscope slide and covered with a cover slip. The sperm were analyzed by fluorescence microscopy (Axiophot Zeiss) using excitation and emission filters at 490/515 nm and 400x magnification. The criteria were based on those set forth by Kumar et al. (2017), whereby sperm with intact plasma membranes exhibited bright green fluorescence, while those with damaged membranes displayed bright red fluorescence. The sperm exhibited green and red fluorescence, indicative of moribund sperm. A minimum of 500 sperm were observed and enumerated.

Statistical analysis

The data were tabulated and presented as mean and standard deviation. The frozen semen quality was subjected to an ANOVA (analysis of variance) analysis at the 95% significance level. The data obtained from the HOS-Test and CFDA-PI were subjected to statistical analysis using the T-test for independent samples with a confidence level of 95%. The correlation between the quality of frozen semen and the integrity of the plasma membrane of Murrah buffalo sperm was subjected to statistical analysis using the Pearson correlation coefficient.

RESULTS AND DISCUSSION

The motility, viability, acrosome integrity, and sperm abnormalities of the Murrah buffalo frozen semen exhibited favorable quality (Table 1). No statistically significant differences were observed in motility, acrosome integrity, or sperm abnormalities of frozen semen among the buffalo bulls ($P>0.05$). The sperm motility values ranged from 57.20±2.01 to 58.78±0.92%. The data in Table 1 illustrate the quality of frozen semen from various Murrah buffalo bulls. According to the Indonesian National Standard guidelines, sperm motility in frozen semen should exceed 40%, with a maximum abnormal sperm count of 20%. The results demonstrate that sperm motility and abnormalities in this study comply with the established quality standards for frozen buffalo semen. Sperm motility is crucial for evaluating sperm fertility (Yaghoobi et al. 2022).

Abnormalities in the shape or structure of sperm, such as an abnormal head, damaged tail, or bent tail, can inhibit sperm function during fertilization. Therefore, sperm morphology evaluation is an important aspect of sperm quality analysis. The prevalence of sperm abnormalities ranged from 6.20±0.73% to 7.10±1.10%. However, the sperm abnormalities in this study were still categorized as relatively minor; thus, the samples still met the standards for insemination. The presence of a considerable number of sperm abnormalities has been demonstrated to have a detrimental impact on fertility (Matabane et al. 2017). Most abnormal sperm shapes in this study were secondary abnormalities, including circular, bent, asymmetrical, or broken tails. These abnormal conditions can inhibit the ability of sperm to move normally toward the egg. Primary abnormalities

Table 1. The mean quality of frozen Murrah buffalo semen from each bull

Name and Bull Code	Sperm Quality ± Standard Deviation (%)			
	Motilities	Viabilities	Acrosome Integrity	Abnormalities
Alex (131118)	57.20±2.01[a]	64.02±2.28 [a]	94.04±1.43[a]	7.10±1.10 [a]
Big Lake (131220)	57.97±1.46[a]	64.87±1.05 [ab]	95.37±0.68 [a]	6.66±0.77 [a]
Caesar (131321)	58.25±1.08 [a]	65.85±1.22 [b]	95.16±1.02 [a]	6.56±1.16 [a]
Millenio (131219)	58.78±0.92 [a]	65.94±0.58 [b]	95.32±0.96 [a]	6.20±0.73 [a]

[a b] Different superscripts in the same column mean a significant difference (P<0.05). A total of 120 samples were obtained from six production batches, with each batch comprising five straws

include macrocephaly, microcephaly, and pear-shaped heads; abnormalities in the spermatogenesis process may cause this. Sperm with head abnormalities are often unable to penetrate the egg membrane effectively.

The highest sperm viability was observed in buffaloes designated Caesar and Millenium, with values of 65.85±1.22 and 65.94±0.58%, respectively. The lowest viability was observed in the buffalo designated as Alex, with a value of 64.02±2.28%. Significant differences (P<0.05) were observed in sperm viability between individual Murrah buffaloes. Differences in sperm viability can be influenced by various internal factors, such as age, reproductive health, and genetics, as well as external factors, such as incorrect techniques in storing frozen semen or improper thawing, which damage sperm and reduce their viability. Nevertheless, sperm viability remains satisfactory. Viability assessment is essential in sperm analysis (Fischer et al. 2020), as it can elucidate damage to the plasma membrane, particularly in the head region, resulting in reduced sperm viability and motility (Dolnik et al. 2019).

The values for acrosome integrity ranged from 94.04±1.43 to 95.37±0.68%. No significant differences (P>0.05) were observed in sperm acrosome integrity between individual Murrah buffaloes. Acrosome integrity was rated excellent, with no significant changes in the acrosomal membrane structure. Ito and Toshimori (2016) elucidate that the sperm head is enveloped by four distinct types of membranes: the plasma membrane, the outer acrosomal membrane, the inner acrosomal membrane, and the nuclear envelope. Olexikova et al. (2019) observed that damage to the acrosome could result in the release of acrosomal enzymes, thereby reducing the sperm's fertilization capacity. As Reis et al. (2016) highlight, the integrity of the acrosome and the maintenance of its enzymes are paramount for the acrosome reaction process.

Figure 1 illustrates the results of the plasma membrane integrity test for each method. The HOS test method identifies two categories of reactions: intact membranes and defective membranes. The CFDA-PI staining method reveals three distinct categories of reactions: intact membrane, damaged membrane, and moribund. The findings of this study indicate that the HOS-Test method exhibited an average of 64.83% intact plasma membranes and 35.17% damaged plasma membranes. The CFDA-PI staining method yielded an average of 58.97% intact plasma membranes, 28.55% damaged plasma membranes, and 12.48% moribund. Table 2 demonstrates that the p-value (P<0.05) indicates statistically significant differences between the mean values of the HOS-Test and CFDA groups.

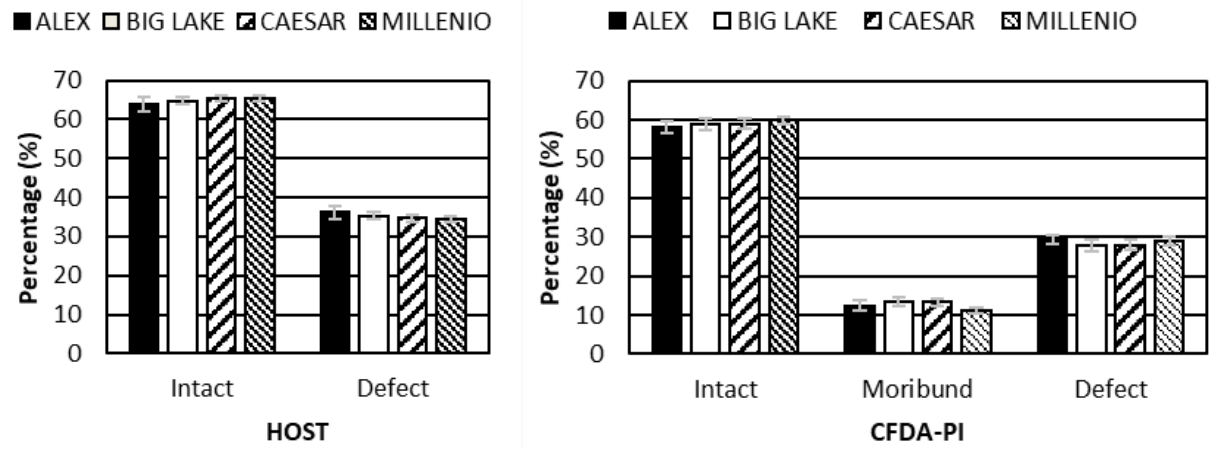

Figure 1. Comparative diagram of the average results of the plasma membrane integrity test of Murrah buffalo semen with HOS-Test and CFDA-PI. HOST: Hypo-Osmotic Swelling Test, CFDA-PI: Carboxyfluorescein diacetate-propidium iodide

Table 2. A comparative analysis of sperm plasma membrane integrity was conducted using the HOS-Test and CFDA-PI dye

Method	Mean	SD	Levene's Test for Equality of Variances		T-test for Equality of Means
			T (T-Test)	P-value	Sig. (2-tailed)
HOS-Test	64.83	1.26	1.44	0.23	0.00
CFDA-PI	58.97	1.40			0.00

HOS-Test: Hypoosmotic swelling test, CFDA-PI: carboxyfluorescence diacetate-propidium iodide. Sig. (2-tailed) indicates a significant difference (P<0.05). A total of 120 samples were obtained from six production batches, with each batch comprising five straws

The results of the sperm membrane integrity analysis, conducted using the HOS-Test method and CFDA staining, demonstrated statistically significant differences (P<0.05). It was postulated that the disparate responses of the two methods were responsible for the considerable discrepancies observed in the percentage of membrane integrity. Figure 1 illustrates that the percentage value of plasma membrane integrity of frozen Murrah buffalo semen, as determined by the HOS-Test method, is higher than that obtained by CFDA-PI staining. The results of the CFDA-PI assessment were categorized into three groups, which led to a lower value for the intact plasma membrane in this evaluation compared to the HOS-Test. In CFDA-PI staining, the condition of a deteriorating plasma membrane can be classified as intact in the HOS-Test. CFDA-PI is highly effective at identifying damage to the sperm plasma membrane. Sperm that are either damaged or dead with compromised plasma membranes were precisely identified using PI, as PI can only enter sperm that have lost membrane integrity. Sperm with intact plasma membranes but internal damage, such as DNA damage, may still show green fluorescence from CFDA, even when nearing death, highlighting this method's sensitivity to internal sperm damage.

The statistical test results indicated a statistically significant positive correlation between sperm motility and the integrity of the sperm plasma membrane, as determined by both the HOS test (r= 0.96) and CFDA-PI staining (r= 0.97). The viability of the sperm was found to be positively correlated with the integrity of the plasma membrane, as determined by CFDA-PI staining (r= 0.99). A significant negative correlation was observed between sperm abnormality and plasma membrane integrity values, as determined by HOS-Test (r= -0.98).

The integrity of the plasma membrane is vital for optimizing sperm function and survival (Bezzera et al. 2018). Damage to the sperm plasma membrane can disrupt cellular metabolism and reduce fertility due to the release of essential enzymes from the acrosome (Olexikova et al. 2019). The HOS-Test method yielded two distinct sperm responses following exposure to the HOS solution: sperm with curled tails and sperm with straight tails (Figure 2). The swelling of the sperm tail in the HOS solution indicates water transport across the membrane, which in turn demonstrates the cell's capacity to maintain equilibrium between its external and internal fluids (Ramu & Jeyendran 2013). Sperm cells in HOS solution undergo biochemical swelling, increasing their volume to achieve equilibrium between the intracellular fluid space and the extracellular environment (Rashedi et al. 2016). The fluid influx into the cell causes a volume change, causing the tail to fold (Figure 2.a).

The HOS test is a straightforward, cost-effective, and user-friendly method for evaluating the integrity of the plasma membrane in diverse species (Zeidan et al. 2018). As Nordhoff (2015) notes, a limitation of this approach is the restricted incubation period that must be allowed to obtain the optimal HOS test response. The assessment of samples using the HOS-Test method is a time-consuming process that requires careful execution. Even if the initial assessment indicates normal cellular function, it is essential to conduct additional tests to ascertain the absence of damage or evidence of infertility (Ramu & Jeyendran 2013).

The sperm examined in this study exhibited three distinct response criteria following exposure to the CFDA-PI dye. These were observed as green cells (intact membrane), red cells (dead or damaged membrane), and green-red cells (moribund). Similarly, Golher et al. (2018) reported comparable findings when evaluating the integrity of sheep sperm crossed with CFDA-PI, which exhibited three distinct response types: intact membrane, moribund, and dead. The dye CFDA can penetrate intact membranes and bind to esterases, thereby enabling the identification of living cells (Dolnik et al. 2019).

The CFDA dye can penetrate the plasma membrane and is hydrolyzed by intracellular esterases, producing fluorescent compounds that emit green fluorescence. In contrast, PI dye can only penetrate dead cells, resulting in the emission of red fluorescence. The study by Kumar et al. (2017) also demonstrated that motile sperm stained with CFDA fluorochrome exhibited widespread cytoplasmic staining while remaining unstained with PI. Given that CFDA is an enzyme-based staining method (enzyme substrate conversion to fluorescent product), and given the issue of time dependence. Figure 3(b) illustrates PI-positive cells, damaged sperm, and

Table 3. Correlation between the quality parameters of frozen semen from Murrah buffaloes

Variable (sperm)		Motilities	Viabilities	Acrosome	Abnormalities	Membrane Integrity	
						HOST	CFDA-PI
Motilities		1	0.94	0.85	-0.99**	0.96*	0.97*
Viabilities		0.94	1	0.78	-0.92	0.84	0.99**
Acrosome		0.85	0.78	1	-0.84	0.78	0.85
Abnormalities		-0.99**	-0.92	-0.84	1	-0.98*	-0.94
Membrane Integrity	HOST	0.96*	0.84	0.78	-0.98*	1	0.88
	CFDA-PI	0.97*	0.99**	0.85	-0.94	0.88	1

**Correlation is significant at the 0.01 level; *Correlation is significant at the 0.05 level. HOST= Hypo-Osmotic Swelling Test, CFDA-PI= Carboxyfluorescein diacetate-propidium iodide. A total of 120 samples were obtained from six production batches, with each batch comprising five straws

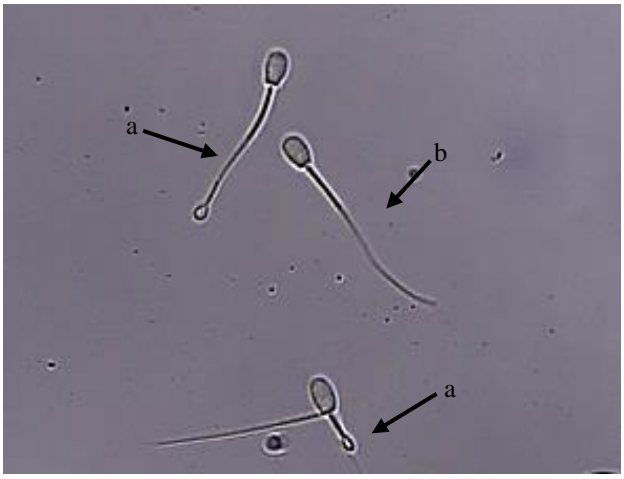

Figure 2. Membrane integrity assessment of Murrah buffalo sperm using the HOS test. Intact plasma membrane (a); defective (b)

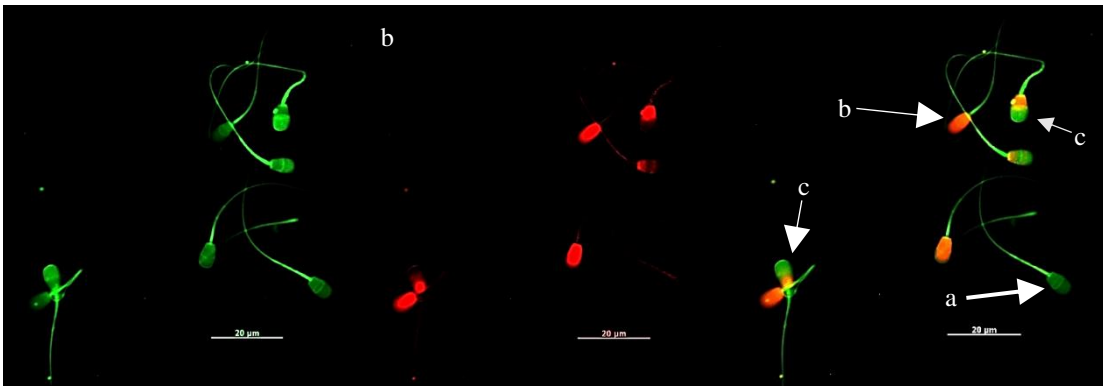

Figure 3. Membrane integrity assessment of Murrah buffalo sperm using CFDA-PI staining. intact plasma membrane (a); defective (b) moribund (c)

fluorescent red. This figure suggests that the PI dye has penetrated and stained cells with damaged plasma membranes.

Sperm exhibiting a dual coloration comprising green and red (Figure 3) are designated as moribund sperm. This phenomenon occurs due to the persistence of esterase enzyme activity within the plasma membranes of sperm cells despite the absence of metabolic activity (Kumar et al. 2017). This residual esterase activity is the underlying cause of both green and red categories among sperm cells. Davey & Guyot (2020) define moribund cells as metabolically inactive despite appearing microscopically intact. Consequently, sperm that have recently undergone apoptosis may still exhibit green coloration due to the continued activity of esterase enzymes, which can result in their misidentification as

cells with intact membranes. As Singh et al. (2016) have observed, moribund sperm display compromised membrane integrity and undergo a transition from a viable state to a moribund one before eventually dying. The plasma membrane of dead sperm loses its ability to resist propidium iodide (PI), resulting in the cells appearing red.

Fluorescent probes can be employed to evaluate the structural integrity and functionality of sperm by binding to and staining specific cellular components (Dolnik et al. 2019). The principal disadvantages of CFDA-PI staining include the high cost of the requisite equipment, the complexity of the fluorescent staining process, and the time required for the subsequent evaluation. Bezerra et al. (2023) demonstrated that cells tagged with CFDA emit fluorescence briefly and then rapidly lose contrast, presenting a significant visualization challenge. Despite these limitations, fluorescence staining remains an advancing technology, and its application in assessing sperm plasma membrane integrity is expected to grow.

The integrity of the plasma membrane serves to protect the sperm's interior physically, regulate the movement of substances and ions that are crucial for metabolic processes, and maintain electrolyte balance. The loss of plasma membrane integrity and reduced sperm motility are pivotal indicators employed to distinguish between dead and living cells. Damage to the plasma membrane can result in metabolic disorders that impair both sperm motility and viability (Gaczarzewicz et al. 2015). In general, higher plasma membrane integrity is associated with improved sperm motility. A breach in the central membrane can impede mitochondrial functionality, reducing adenosine triphosphate (ATP) production and declining sperm motility (Barbagallo et al. 2020).

The results of this study indicate a positive correlation between the integrity of the sperm plasma membrane and the viability of the sperm. The integrity of the sperm plasma membrane is a critical factor influencing sperm viability, particularly in the head region (Dolnik et al. 2019). Prior research has indicated that the assessment of sperm viability may serve as an indicator of plasma membrane structure integrity (Sukmawati et al. 2014). Moreover, a negative correlation has been observed between sperm abnormalities and plasma membrane integrity, indicating that higher sperm abnormalities are associated with lower plasma membrane integrity. As Garcia-Vazquez et al. (2016) have observed, sperm morphology analysis may prove a valuable means of detecting changes related to sperm membrane integrity. Matabane et al. (2017) underscored the importance of evaluating sperm morphology and plasma membrane integrity for predicting sperm fertility.

The integrity of the plasma membrane can be evaluated using either the HOS-Test or the CFDA-PI test, according to the specific requirements and perspectives of the researcher. The HOS test is a more cost-effective and uncomplicated procedure, utilizing a straightforward instrument that can be performed in any setting. In contrast, the CFDA-PI test is a more complex procedure that requires the input of a specialist and the use of costly equipment, such as fluorescence microscopes, which are typically only available in specialized laboratories. However, this issue can be addressed by providing better training or collaborating with other laboratories. CFDA-PI is more sensitive in detecting damaged or moribund sperm than the HOS Test.

Furthermore, the HOS-Test may yield positive results for moribund sperm, which could lead to erroneous conclusions. Conversely, the CFDA-PI test is more precise, offering three distinct categories of results and exhibiting a robust positive correlation with sperm motility and viability. The discrepancy in accuracy between the two methods is approximately six percent.

CONCLUSION

This study's findings indicate notable differences between the HOS-Test and CFDA assessments of frozen Murrah buffalo semen, as evidenced by the statistical analysis. Both methods are appropriate for detecting plasma membrane integrity; however, selecting the most appropriate method can be adjusted according to the specific requirements of the situation. The HOS-Test assessment is a rapid, cost-effective, and practical method employed in laboratory and field settings. CFDA-PI staining may be employed to conduct a more accurate and precise assessment of sperm plasma membrane integrity, thereby providing a more detailed and accurate basis for determining the quality of frozen semen.

ACKNOWLEDGEMENT

The authors would like to express their gratitude and highest appreciation to the National Research and Innovation Agency (BRIN) technician and staff for their invaluable assistance in facilitating the research. This study was financially supported by the Riset dan Inovasi untuk Indonesia Maju (RIIM) Batch 3 Partnership between BRIN and LPDP through project number 12/II.7/HK/2023.

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