The Quality of Indo-Pacific Bottlenose Dolphin (*Tursiops aduncus*) Sperm Following Liquid-storage in Low Temperature

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Abstract

Indo-Pacific bottlenose dolphin (Tursiops aduncus) is a marine mammal that lives in relatively small populations. The geographic ranges of this species are susceptible to the effects of human activities, thereby necessitating conservation efforts to prevent extinction. Therefore, this study aimed to evaluate the daily quality of dolphin sperm after several days of refrigeration. The sperm of two male dolphins were stored at 4^{o}C for 4 days, and the quality was observed daily to determine the motility, viability, membrane integrity, and sperm abnormalities. Sperm samples were divided into four groups, consisting of two centrifuged followed by the removal of seminal plasma, and two groups without centrifugation, containing 100x10^{6} and 200x10^{6} sperm/ml each. After liquid storage, the motility of sperm was 63-75% with no significant reduction in the first 3 days. Sperm viability following storage was 65-75% and the percentage with abnormal morphology ranged from 2-6%. Furthermore, there was no significant increase in abnormal morphology of sperm on any day of storage for 3 days. Sperm membrane integrity was 36-49%, with no significant reduction in the membrane integrity in the first 2 days. There was no significant difference in sperm quality, although centrifugation and removal of seminal plasm had a slight effect. The results of this study showed that Indo-Pacific bottlenose dolphin sperm could be stored for a short period as liquid storage while maintaining a quality that allows for future use.

Keywords: *dolphin, sperm, preservation, motility, viability, liquid-storage*

Introduction

Indo-Pacific bottlenose dolphin (*Tursiops aduncus)* is a marine mammal (cetacean), which is one of the biological resources in marine waters of Indonesia. This marine mammal plays an important role in the ecosystems (Valls *et al*., 2015) by significantly maintaining stability and integrity of the holocoenosis (Bowen and Role, 1997). Dolphin is a species of bottlenose dolphin with a smaller body size and a longer snout than *Tursiops truncates* and *Tursiops australis*. This species are distributed in warm and tropical waters in Indo-Pacific, specifically in coastal areas and shallow estuaries as well as shallow reef complexes (Jefferson *et al*., 2015). Dolphin lives in relatively small populations or herds with geographic range that is vulnerable to impacts from human activities (Gray *et al*., 2018), such as fishing, maritime traffic, pollution, and degradation of coastal ecosystems (López, 2012). These human activities continuously affect the habitat (Reynolds, *et al*., 2009) as well as the health and population of the species (Pace *et al*., 2015). According to the IUCN (International Union for Conservation of Nature) Red List of Threatened Species, dolphin was classified as "Near Threatened" or "At Risk of Extinction" (Braulik *et al*., 2019) when no sustainable conservation measures were taken. Therefore, efforts to protect this marine mammal have received great attention.

Conservation measures aimed to protect a species in the natural habitat (*in situ*) and also maintain a sufficient captive population (*ex situ*) for the purpose of reintroduction. To achieve this objective, conservation and captive breeding programs needs to be implemented. Breeding programs for endangered animals, including marine mammals, can be optimized through the use of artificial insemination (AI) technology. However, the use of AI requires the availability of sperm cells that

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can be used at any time (sperm bank). In livestock or other animals, methods were developed for storing sperm in liquid form (preservation) at a temperature of 4°C (Citraesti *et al*., 2021; Khye *et al*., 2021; Siregar *et al*., 2023) or in frozen form (cryopreservation) (Karja *et al*., 2016; Baharun *et al*., 2023; Putri, *et al*., 2023). A fundamental and comprehensive understanding of sperm characteristics and the interspecies variations is important for the success of sperm preservation, particularly information on spermatogenesis and sperm biology of species-specific reproductive data (Van der Horst *et al*., 2018).

Preserving sperm through low-temperature storage offers a potential solution for ex-situ dolphin breeding programs in protected areas, particularly where access to frozen sperm is restricted or unavailable. This method can help facilitate the conservation and management of dolphin populations. Lowering the storage temperature below body temperature is a common strategy to reduce cell metabolism (Rauen and Groot, 2002) and extend storage time. However, hypothermic conditions can have other detrimental effects on sperm, particularly on sodium homeostasis (Murphy *et al*., 2015). For example, the Na+/K+ pump reduces the activity at 5°C by increasing intracellular Na+ levels (Murphy *et al*., 2015; Vishwanath and Shannon, 2000). Similarly, during the cryopreservation process, various types of damage are experienced by all aspects of cells, the tissue anatomy, and physiology, resulting in a reduction in cell or tissue function (Parks and Graham, 1992). Sperm cryopreservation causes various physical, biochemical, and oxidative damages to sperm membranes, leading to reduced viability and fertilizing capacity, as well as loss of motility, plasma membrane function, and sperm acrosome integrity (Watson, 2000). There is no standard protocol for the preservation and cryopreservation of dolphin sperm with satisfactory results. The information about the daily quality of dolphin ejaculate in terms of motility, viability, membrane integrity and abnormality after preservation is very limited. Therefore, this study aimed to evaluate the daily quality of dolphin sperm after several days of refrigeration. The information from this study may provide stored sperm with a simple method to maintain the availability of dolphin sperm in field laboratories, specifically for artificial insemination of female bottlenose dolphin.

Materials and Methods

Animal

Two adult male dolphins were used for this study, aged 11 and 18 years old, with a body weight of approximately of 110 kg and 105 kg, respectively. Both males were kept in captivity at the Wersut Seguni Indonesia Animal Park, with the same environmental, nutritional, and reproductive management. Dolphins live in outdoor pools with seawater at a temperature of 24-32°C. This study was approved by the Animal Ethics Committee of the School of Veterinary Medicine and Biomedical Sciences of IPB University (092/KEH/SKE/VIII/ 2023).

Sperm collection and processing

Sperm samples of trained male dolphins were collected according to the procedures described by Ruiz-Diaz *et al*. (2020) with minor modifications. The dolphin swims voluntarily with belly above the water level of the pool and were made to stick out the penis by hand signals. After complete extrusion, manual stimulation was applied to trigger ejaculation. The ejaculated sperm was then collected directly in a bottle and immediately taken to the laboratory. Only sperm samples with sperm motility of more than 80% were used for the experiment.

The collected sperm sample was divided into four groups, and TEST-yolk-buffered extender was added until to a final concentration of 100 x 10^6.ml^{-1} and 200 x 10^6.ml^{-1} (NC-100 and NC-200 groups). In other groups, sperm was centrifuged at 3000 rpm for 15 min and extender was added until the concentration was 100 x 10^6.ml^{-1} and 200 x 10^6.ml^{-1} (C-100 and C-200 groups) after the seminal plasma was removed. The medium used in this study was TEST-yolk-buffered extender (cat. 90129, FUJIFILM Irvine Scientific, Inc), containing 176 mM TES, 80 mM tris, 9 mM dextrose, 10 µg.ml^{-1} gentamicin sulfate, and 20% (v/v) Heat-inactivated egg yolk. All samples were stored in the refrigerator at 4°C for 4 d. Sperm quality during storage was assessed daily for sperm motility, viability, and plasma membrane integrity up to day four (D-4).

Evaluation of sperm motility

The progressive motility of sperm was examined subjectively. For evaluation, 10 µl of sperm was dropped onto a microscope slide and covered with a glass coverslip. The samples were then placed on the microscope stage and assessed from five fields of view.

Evaluation of sperm viability

Sperm viability was assessed after the samples were stained with Eosin-Nigrosin dye. A total of 10 µl of sperm sample and 40 µl of Eosin-Nigrosin were mixed on a microscope slide. The samples were prepared by smearing on the microscope slide and drying on a small fire of Bunsen burner. Observations

under a microscope with 400x magnification showed that live sperm do not absorb color, while dead sperm absorb color. In this study, the total number of sperm counted was 200.

Evaluation of sperm membrane integrity

The integrity of sperm membrane was examined using hypoosmotic swelling test (HOS). A 20 µl sperm sample was diluted with 80 µl HOS solution and stored at 37°C for 30 min. Furthermore, a 10 µl sperm sample was dropped into a microscope slide covered with a coverslip, and analyzed under a microscope at 400x magnification. Two hundred sperm were counted to determine those with circular (plasma membrane intact) and straight tails (plasma membrane not intact).

Evaluation of sperm abnormality

The abnormality of sperm morphology was assessed by Eosin-Nigrosin staining. Microscopic observation of abnormalities was performed on sperm that had abnormal shapes. The samples were observed under a microscope with 400x magnification in five different fields of view, with at least 200 sperm.

Data analysis

Data were presented as percentage ± standard deviation (SD) and semen characteristics were obtained from a minimum of three replicates. Sperm trait data were statistically analyzed by two-way ANOVA (Analysis of Variance) using SAS 9.4. Duncan's Multiple Range Test (DMRT) was performed for treatments with significant differences.

Result and Discussion

Sperm motility

Motility of Indo-pacific bottlenose dolphin sperm after preservation/cooling at 4°C is shown in Figure 1. Daily observation of sperm motility after 4 days of storage ranged from 65-70% and 63-75% for Male-1 and Male-2, respectively. A significant decrease in sperm motility occurred on the 4th day of storage in all groups, both Male1- and Male-2. The only exception was the NC-200 group in Male-1, in which sperm motility had already decreased on the 3rd day of storage. There were also no significant differences in sperm motility in Male-1 between the groups on any day of storage. However, the motility of sperm from Male-2 was better in the group stored without centrifugation (P<0.05). In this study, two groups of sperm concentrations, namely 100x10^6 and 200x10^6 were used to assess the effect of concentration on sperm motility. The results showed that the motility of sperm stored at 4°C did not experience a significant decrease until the 3rd day of storage. However, sperm motility in all storage groups continued to decrease until the 4th day (P<0.05).

Sperm viability

In Male-1, sperm viability after 4 days of storage ranged from 65-73%, as shown in Figure 2. A decrease in sperm viability occurred on the 3rd and 4th day in NC-100 and NC-200 groups, respectively, as evidenced by P<0.05. In the centrifuged groups (C-100 and C-200), viability was significantly decreased on the 4th day. No significant difference in sperm viability was observed between the treatment groups (P>0.05) except on the 1st day when sperm viability from NC-100 was higher (P<0.05). Sperm viability was still maintained above 65% in all treatment groups until the 4th day of storage. In Male-2, sperm viability after 4 days of refrigeration was between 71-79%. A decrease in sperm viability occurred on the 3rd and 4th day (P<0.05) in NC-100 and C-100 groups, respectively. NC-200 and C-200 groups did not differ until the 4th day (P>0.05) of storage. No significant difference was found between treatment groups on any of the 1st day (P>0.05). On the 2nd day, sperm viability in the groups stored without centrifugation was higher, as evidenced by P<0.05. On the 3rd day, differences in sperm viability were observed between NC-200, C-100, and C-200 groups (P<0.05). Sperm viability was higher till the 4th day in NC-100 group than in C-200 (P<0.05). Sperm viability was still maintained above 65% in all treatment groups until the 4th day of storage.

Sperm membrane integrity

Sperm membrane integrity at 4 days of storage ranged from 36-47% and 42-49% for Male-1 and Male-2, respectively (Figure 3.). In Male-1, a decrease in sperm membrane integrity occurred on the 3rd, 4th, and 2nd day (P<0.05) in the groups without centrifugation (NC-100 and NC-200), C-100, and C-200 (P<0.05), respectively. Significant differences were found in membrane integrity of Male-1 in NC-200 and C-100 (P<0.05) on the 1st day. On the 2nd and 3rd days, there were no significant differences in membrane integrity between groups. However, on the 4th day, membrane integrity was lower in the non-centrifugation group, as evidenced by P<0.05. In Male-2, this study observed a decrease in sperm membrane integrity in the centrifuged groups on the 4th day of storage. There was no significant difference in sperm membrane integrity between treatment groups on the 1st and 2nd days of storage (P>0.05). However, on the 3rd day, the lowest membrane integrity was found in NC-200 (P<0.05). On the 4th day, sperm membrane integrity in C-100 was higher,

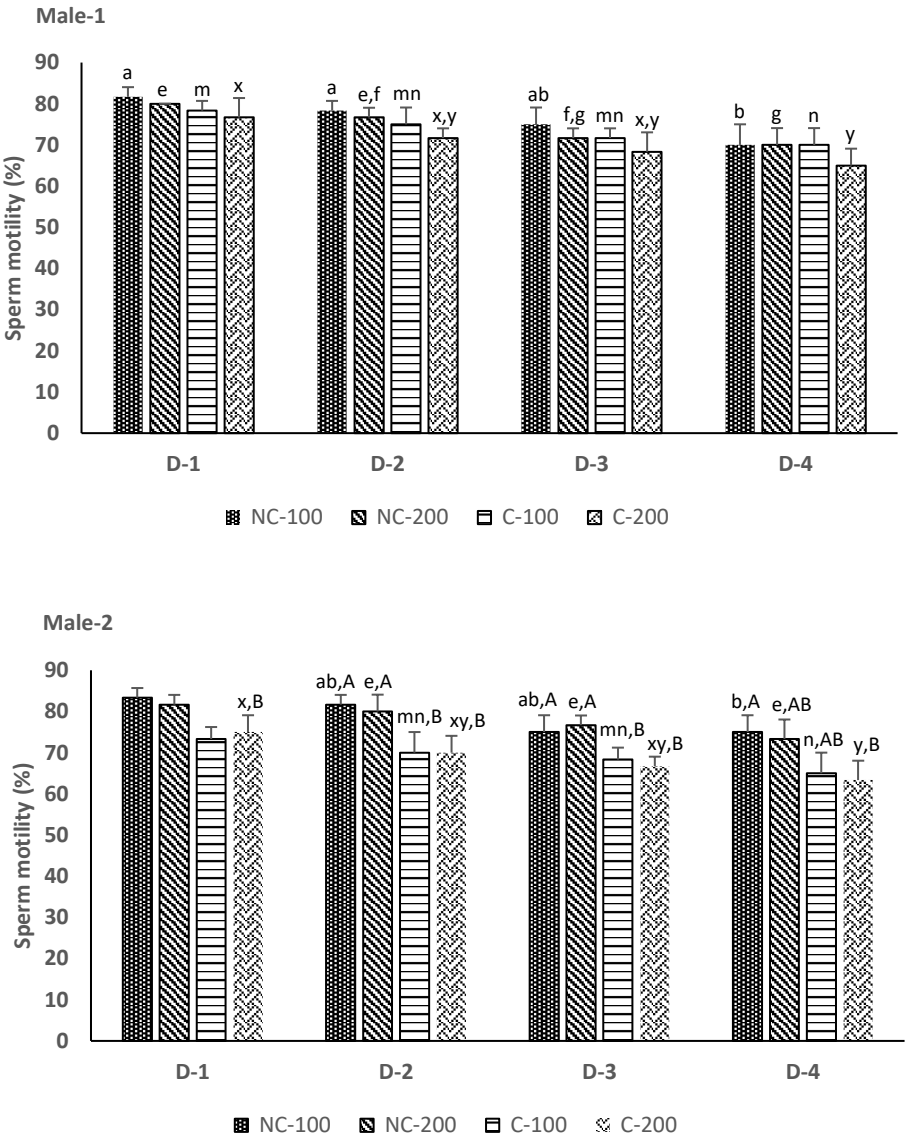

Figure 1. Motility of Indo-Pacific Bottlenose dolphin sperm (*Tursiops aduncus*) in Test-Yolk-buffered extender after storage at 4°C for several days. Semen was stored without centrifugation with concentrations of 100 x 10^6 (NC-100) and 200 x 10^6 (NC-200) or centrifuged with concentrations of 100 x 10^6 (C-100) and 200 x 10^6 (C-200). In each end point, bars with different letters (a-b, e-g, m-n, and x-y) are significantly different for NC-100, NC-200, C-100, and C-200, respectively (P< 0.05). In groups, bars with different letters (A-B) are significantly different for NC-100, NC-200, C-100, and C-200, respectively (P< 0.05).

as evidenced by P<0.05. This result showed the possible effect of centrifugation or removal of seminal plasm on sperm membrane integrity.

Sperm abnormality

Abnormal sperm morphology during 4 days of storage was 2-6%, as shown in Figure 4. In Male-1, a slight increase in abnormal sperm morphology was found on the 3rd day of storage in all groups, except in C-100 group which occurred on the 4th day (P<0.05). There was no significant difference in abnormal sperm morphology between the treatment groups (P>0.05) except on the 1st day of storage (NC-200 vs. C-100 groups). In Male-2, no differences in abnormal morphology were observed in NC-100 and C-100 groups (P>0.05) until the 4th day of storage. A slight increase in abnormal sperm morphology was observed on the 4th day in NC-200 and C-200 groups (P<0.05). Meanwhile, there was no significant difference in abnormal sperm morphology between the treatment groups during 3 days of storage (P>0.05)

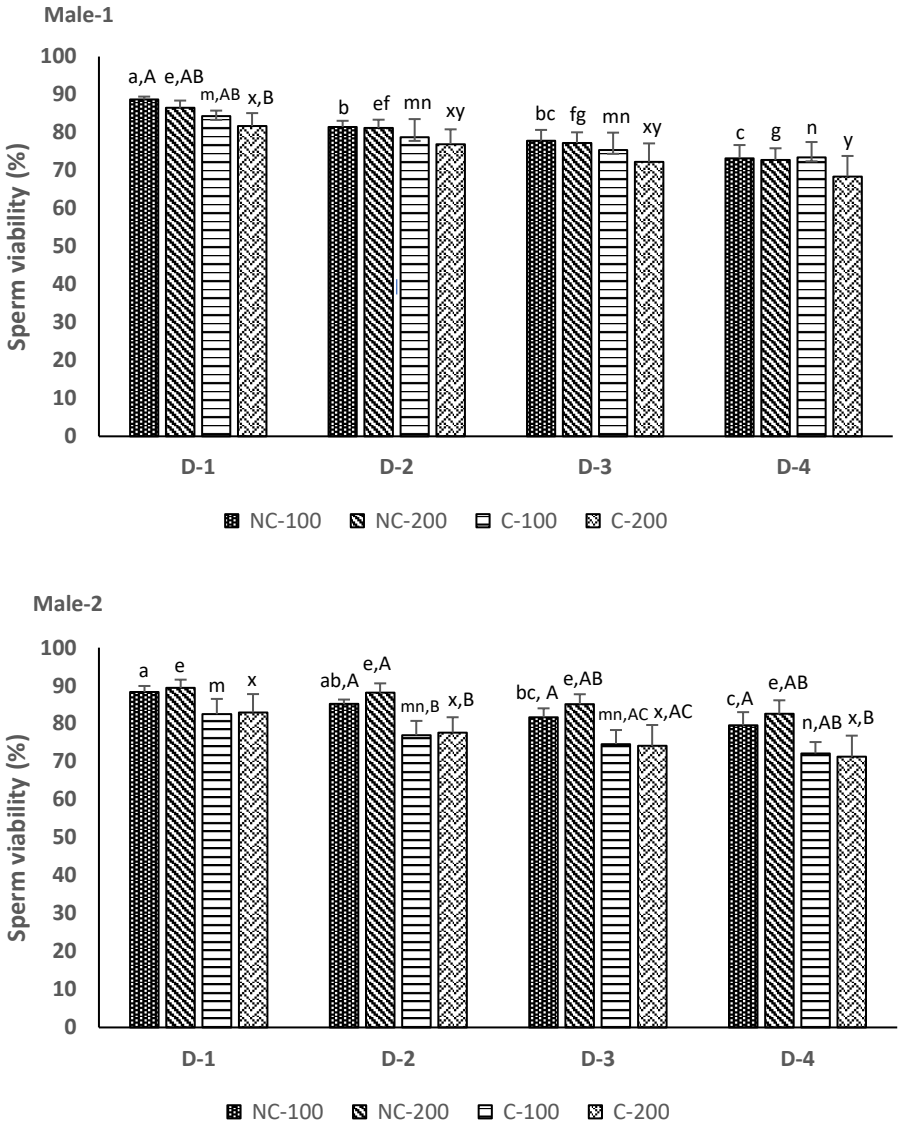

Figure 2. Viability of Indo-Pacific Bottlenose dolphin sperm (*Tursiops aduncus*) in Test-Yolk-buffered extender after storage at 4°C for several days. Semen was stored without centrifugation with concentrations of 100 x 10^6 (NC-100) and 200 x 10^6 (NC-200) or centrifuged with concentrations of 100 x 10^6 (C-100) and 200 x 10^6 (C-200). In each end point, bars with different letters (a-c, e-g, m-n, and x-y) are significantly different for NC-100, NC-200, C-100, and C-200, respectively ($P < 0.05$). In groups, bars with different letters (A-B) are significantly different for NC-100, NC-200, C-100, and C-200, respectively ($P< 0.05$).

and an increase in abnormality was found on the 4th day ($P<0.05$).

This study is the first to report the effect of liquid storage of Indo-Pacific bottlenose dolphin sperm on daily quality after 4°C storage in a refrigerator for 4 days. The results showed that the quality of dolphin sperm generally decreased with increasing storage time. A decline in sperm quality during storage can occur due to several factors, including the availability of energy required for sperm metabolism. Motility is one of the most important parameters in fertilization because sperm must cross the uterus to reach the site of fertilization during natural mating (Bearden *et al*., 2004). Sperm motility after storage could be affected by sperm concentration during storage (O'Hara *et al*., 2010; Ruiz-Diaz *et al*., 2020), where high sperm concentration caused low motility due to high collision activity. The higher the concentration in sperm storage, the greater the opportunity for collisions between sperm (Van der Horst *et al*., 2018).

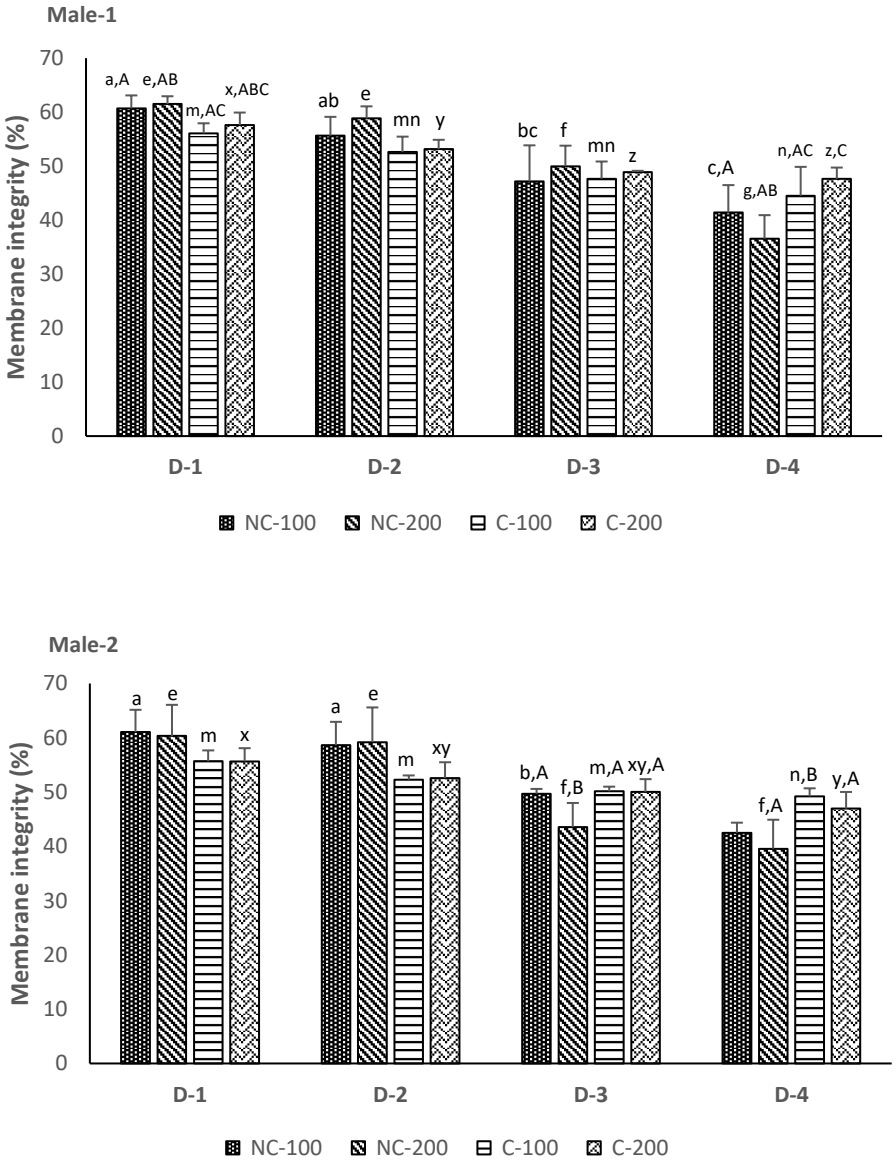

Figure 3. Membrane integrity of Indo-Pacific Bottlenose dolphin sperm (*Tursiops aduncus*) in Test-Yolk-buffered extender after storage at 4°C for several days. Semen was stored without centrifugation with concentrations of 100 x 10^6 (NC-100) and 200 x 10^6 (NC-200) or centrifuged with concentrations of 100 x 10^6 (C-100 group) and 200 x 10^6 (C-200). In each end point, bars with different letters (a-c, e-f, m-n, and x-z) are significantly different for NC-100, NC-200, C-100, and C-200 treatments, respectively (P< 0.05). In groups, bars with different letters (A-B) are significantly different for NC-100, NC-200, C-100, and C-200, respectively (P< 0.05).

The decline of sperm motility in this study is consistent with a previous report in bottlenose dolphins (Robeck and O'Brien, 2004), who reported that cryopreserved sperm had reduced motility after thawing when stored at a concentration of 200 x 10^6 sperm compared to 100 x 10^6. Moreover, high sperm concentration may result in high metabolic output, which related to oxidative stress (Agarwal *et al*., 2014), and the formation of endogenous free radicals/ROS is the main cause of reduced motility in sperm storage (Kasimanickam *et al*., 2006; Gundogan *et al*., 2010). High metabolic output also causes the decrease in pH, as it has been reported that decreasing pH during storage for several days could also reduce sperm motility (Liu *et al*., 2016; Kumar *et al*., 2024). However, the hypothermic condition during storage decreased the metabolic rate, which may contribute to the prolonged survival of spermatozoa (Vishwanath and Shannon, 2000).

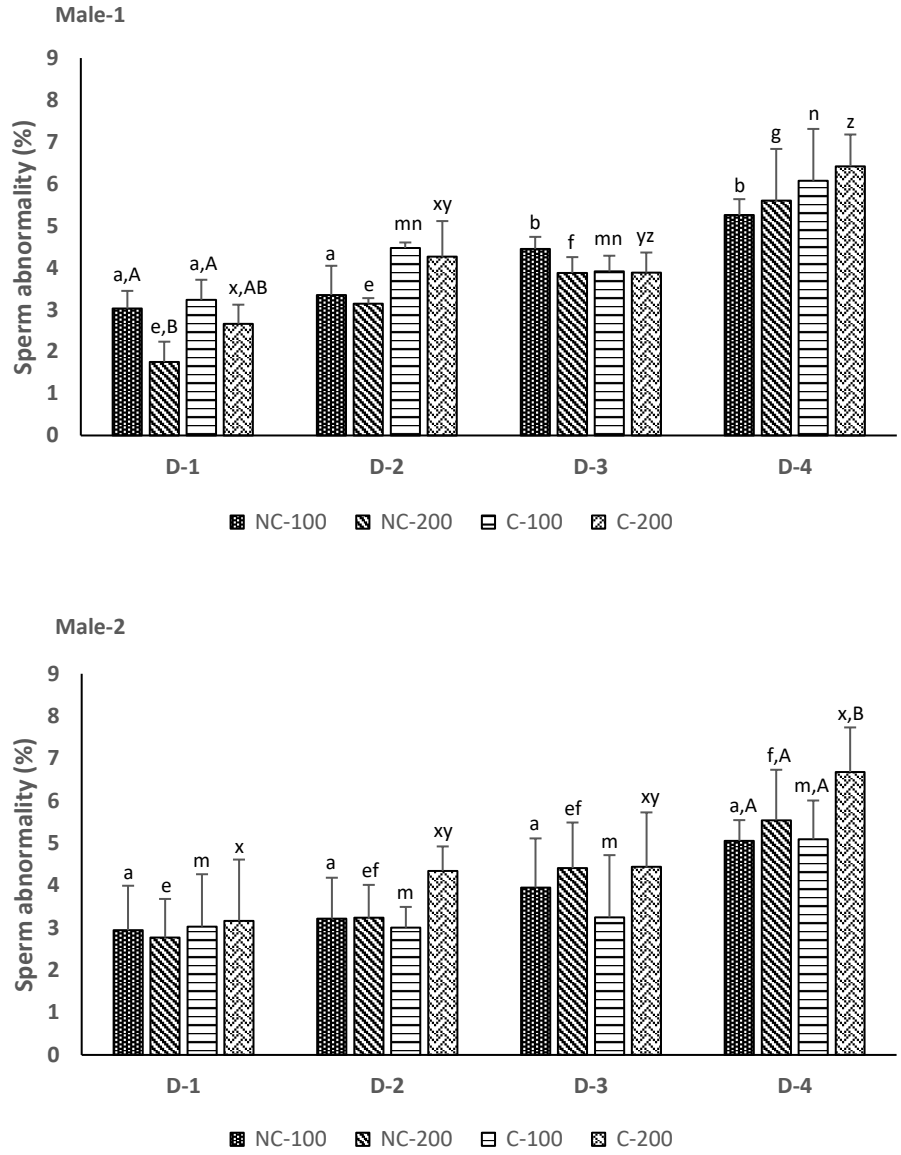

Figure 4. Abnormality of Indo-Pacific Bottlenose dolphin sperm (*Tursiops aduncus*) in Test-Yolk-buffered extender after storage at 4°C for several days. Semen was stored without centrifugation with concentrations of 100 x 10^6 (NC-100) and 200 x 10^6 (NC-200) or centrifuged with concentrations of 100 x 10^6 (C-100) and 200 x 10^6 (C-200). In each end point, bars with different letters (a-b, e-f, m-n, and x-z) are significantly different for NC-100, NC-200, C-100, and C-200, respectively (P< 0.05). In groups, bars with different letters (A-B) are significantly different for NC-100, NC-200, C-100, and C-200, respectively (P< 0.05).

Seminal plasma is a complex buffer fluid that contains a wide variety of proteins essential for sperm transport, protection, and maturation. In spermatozoa, most proteins bind to the sperm surface via exosomes (epididymosomes and prostosomes), which can modulate sperm function (Samanta *et* al., 2018; (Fuentes-Albero *et al.,* 2021), and acts as a buffer as well as a medium for sperm motility (Agarwal *et al*., 2014). The presence of seminal plasma has been reported to affect sperm quality during storage (Takenaka *et al*., 2013; Höfner *et al*., 2000). Although seminal plasma was important in preventing spontaneous capacitation, studies showed that sperm resuspended in an extender after removal of seminal plasma could improve sperm performance (Cheng *et al*., 2022). However, the use of extenders as a complete replacement for seminal plasma during fluid storage is still controversial (Höfner *et al*., 2000), as shown in this study, the removal of seminal plasma prior sperm storage gave varied sperm quaity among dolphins.

The ability of sperm to survive is also another important factors in determining sperm quality. Sperm viability is strongly influenced by plasma membrane integrity as the role of protecting the organelles of sperm and electrolyte transport for metabolism. In this study, the viability and membrane integrity of sperm were gradual decreased during storage. In general, plasma membrane undergoes a phase transition at about 4–10 °C, leading to fragility and loss of integrity (Sieme *et al*., 2015). This structural change makes sperm more susceptible to free radicals when in contact with oxygen (Sankai *et al*., 2001; Kadirvel, *et al*., 2009). Damage to the plasma membrane affects the physiological function and metabolism of sperm, resulting in mortality. As shown in Figure 2. sperm viability gradually decreased during storage for 4 days, although there was no significant difference in the daily percentage of viability in all groups

The integrity of dolphin sperm membrane steadily decreased during storage and was significant on the 3rd day (Figure 3.). This decrease occurred more rapidly in the group that was centrifuged and had the seminal plasma removed The effect of centrifugation or seminal plasma reduction has been reported to affect the quality of sperm stored in the liquid state (Höfner *et al*., 2000) and in dolphins (Takenaka *et al*., 2013). In addition to phase transition effects (Sieme *et al*., 2015), plasma membrane damage during sperm storage may also be associated with oxidative stress (Peris-Frau *et al*., 2020). The integrity of sperm membrane phospholipids during temperature changes alters the lipid-protein, lipid-carbohydrate, and protein-carbohydrate interactions required for membrane activity. These changes caused a reduction in disulfide bonds between membrane proteins, peroxidation of phospholipids, and modification of sperm glycocalyx, resulting in impaired sperm function. As a result, the sperm membrane becomes fragile and loses semipermeability..

Abnormal sperm morphology, such as a broken or severely coiled tail may be related to sperm quality in general, such as viability and motility. Loss or abnormalities in the tail cause the inability to move progressively to reach the fertilization site. Abnormality of Indo-Pacific bottlenose after storage for 4 days was up to 6%, which still below the acceptable threshold for artificial insemination in livestock. Suhardi *et al.* (2019) reported that bull sperm was able to maintain an abnormal level at < 25% for 3 days of storage. According to Da Costa *et al.* (2016), sperm abnormalities that reached 25% did not affect fertility. In this study, a difference in the degree of sperm abnormality was observed on day 1 in Male-1 (Figure 4.). However, the abnormal increase in sperm count in Male-2 occurred only on the 4th day in the centrifuged group. Centrifugation and removal of seminal plasma tend to cause an increase in dolphin sperm abnormalities but do not cause a significant reduction in sperm quality in terms of motility, viability, and membrane integrity. Both males have different sensitivities but the result showed that dolphin sperm can be stored in liquid with a fairly high percentage of normal sperm whereas abnormal increase is below 25%. Based on sperm motility, viability, and membran integrity this study concluded that dolphin sperm abnormalities did not increase significantly during 4 days of storage at 4°C.

Short-term storage of bottlenose dolphin sperm in the form of liquid sperm is important in providing sperm pools for artificial insemination, specifically in areas that have limited facilities for storing frozen sperm. This is also important as there are no institutions that provide frozen dolphin sperm, even for conservation purposes. The study of dolphin sperm motility, sperm viability, abnormal morphology, and membrane integrity provides an opportunity for storing sperm ejaculate for several days without showing a significant decrease in quality.

Conclusion

In conclusion, this study was the first to investigate the effect of liquid storage of Indo-Pacific bottlenose dolphin sperm on daily quality after 4 days stored at 4°C in a refrigerator. The results showed that Indo-Pacific bottlenose dolphin sperm could be stored for a short period while maintaining a quality allowing for artificial insemination programs in the future.

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