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Research

Liquid Semen Characteristics of Indo-Pacific Bottlenose Dolphins (*Tricops aduncus*) after storage at 5°C

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ABSTRACT

Indo-Pacific bottlenose dolphins are potential models for the development of semen preservation in marine mammals. Lecithin-based commercial diluent is used for preservation of Indo-Pacific bottlenose dolphin liquid semen. This study aims to evaluate the characteristics of liquid semen of bottlenose dolphins after storage at 5°C in lecithin-based commercial diluents. Fresh semen from two 11,5 and 18-year-old Indo-Pacific bottlenose dolphins was collected once a week for 3 replicates. Fresh semen was evaluated microscopically and macroscopically. Semen with more than 70% sperm motility was divided into four tubes, each with and without centrifugation treatment. Semen was divided into concentrations of 100×10^6 ml^{-1} and 200×10^6 ml^{-1}, then stored at 5°C for 4 days. Semen was stored in 15 mL tubes and kept in a refrigerator at 5 °C for 4 days. Evaluation was done every 24 hours, including motility, viability, and plasma membrane integrity (PMI) of sperm. Results showed a significant decrease (P < 0,05) in sperm quality every 24 hours of examination in all treatments. Liquid semen quality was maintained with motility ≥ 40% until day 2 after semen collection. Liquid semen preservation for Indo-Pacific bottlenose dolphin sperm can be done for a short storage period of 48 hours in all treatments with lecithin-based commercial diluent.
Keywords: Semen quality, commercial extender, liquid semen, bottlenose dolphin

ABSTRAK

Lumba-lumba hidung botol Indo-Pasifik merupakan model potensial untuk pengembangan preservasi semen pada mamalia laut. Pengencer komersial berbasis lesitin digunakan untuk preservasi semen cair lumba-lumba hidung botol indo-pasifik. Penelitian ini bertujuan untuk mengeavaluasi karakteristik semen cair lumba-lumba hidung botol pasca penyimpanan pada suhu 5 °C dalam pengencer komersial berbasis lesitin. Semen segar dari dua lumba-lumba hidung botol indo-pasifik berusia 11,5 dan 18 tahun dikoleksi satu minggu sekali sebanyak 3 kali ulangan. Semen segar dievaluasi secara makroskopis dan mikroskopis. Semen dengan motilitas sperma lebih dari 70% dibagi menjadi empat tabung, masing-masing dengan dan tanpa perlakuan sentrifugasi. Semen dibagi menjadi konsentrasi 100×10^6 ml^{-1} dan 200×10^6 ml^{-1}, kemudian disimpan pada suhu 5 °C selama 4 hari. Evaluasi dilakukan setiap 24 jam meliputi motilitas, viabilitas, dan membran plasma utuh (MPU) spermatozoa. Hasil penelitian menunjukkan adanya penurunan yang signifikan (P < 0,05) pada kualitas sperma setiap 24 jam pemeriksaan pada semua perlakuan. Kualitas semen cair dipertahankan dengan motilitas ≥40% hingga hari ke-2 setelah penampungan semen. Preservasi semen cair sperma lumba-lumba hidung botol indo-pasifik dapat dilakukan dalam jangka waktu penyimpanan yang singkat yaitu 48 jam pada semua perlakuan dengan pengencer komersial berbasis lesitin.
Kata kunci: Kualitas semen, pengencer komersial, semen cair, lumba-lumba hidung botol

INTRODUCTION

Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) are marine mammals that form part of the biodiversity of Indonesia's marine resources. Dolphins play an important role in the marine ecosystem (Valls et al., 2015). However, the existence of dolphins is threatened by human activities such as pollution, marine traffic, and coastal ecosystem degradation, which affect their habitat and population health status (Pace et al., 2015). This is exacerbated by threats to the decline of the Indo-Pacific bottlenose dolphin population, such as being caught in fishing nets and hunted for their meat or used as bait (Braulik et al., 2018; Mintzer et al., 2018). Based on their conservation status, Indo-Pacific bottlenose dolphins are listed in Appendix II of CITES (Convention on International Trade in Endangered Species of Wild Flora and Fauna), which restricts the movement of animals or their products between countries. In addition, according to the IUCN (The International Union for Conservation of Nature), Indo-Pacific bottlenose dolphins are classified as "near threatened" or close to the threshold of endangered if no sustainable conservation measures are taken.

Conservation measures for Indo-Pacific bottlenose dolphins can be carried out, among other things, by increasing understanding and capabilities regarding ex situ breeding with the help of reproductive technology. Research related to reproductive technology, such as sperm preservation in dolphins, has not been widely conducted in Indonesia. Sperm preservation is used as a tool to help provide quality semen for artificial insemination. In dolphins, insemination is performed 28–32 hours after the onset of the luteinizing hormone (LH) surge (Robeck et al., 2013). In insemination treatments with locations different from the semen storage site, sperm preservation helps maintain semen quality during transportation. In addition, dolphin sperm preservation needs to consider seminal plasma and sperm concentration. Seminal plasma has beneficial effects on animals such as cattle and sheep, but is harmful and toxic to horses (Kalashnikov et al., 2019). Dolphin seminal plasma has been reported to have no significant effect on sperm viability (Takenaka et al., 2013). Suboptimal sperm concentration when stored for several days is associated with greater changes in structural and functional variables of sperm in male sheep. In dolphins, liquid semen has been reported to be stored for short periods with sperm function maintained when stored at a concentration of 100 x 10^6 ml^{-1} (Ruiz-Díaz et al., 2020).

A basic and comprehensive understanding of sperm characteristics and variations between species is essential to support successful sperm preservation. Information about spermatogenesis and sperm biology is important reproductive data, especially species-specific data. Optimization of sperm preservation methods is necessary to ensure that the developed methods are capable of maintaining sperm viability. Therefore, this study evaluated the characteristics of bottlenose dolphin semen after storage at 5 °C in a commercial lecithin-based diluent to provide high-quality semen that conservation institutions can use.

MATERIALS AND METHODS

Fresh semen source

Fresh semen was obtained from two Indo-Pacific bottlenose dolphins aged 11.5 and 18 years, weighing 105 and 110 kg. Both dolphins were intensively cared for in two separate pools measuring 9 meters long and 5.4 meters wide, and 14 meters long and 4.8 meters wide, respectively. Both pools were 3.5 meters deep. They were fed twice a day with 3.2–3.5 kg of mackerel in the morning and afternoon. The pools are cleaned daily and the water is changed periodically. The pools are checked routinely every day to determine the pH, salinity, and chlorine levels. This research has been approved by the Animal Ethics Committee of the School of Veterinary Medicine and Biomedical Sciences, IPB University (092/KEH/SKE/VIII/2023).

Semen Collections and Evaluations

Semen samples were collected once a week from trained male dolphins. After collection, the samples were transferred to 15 mL tubes and stored at 21 °C, then evaluated macroscopically and microscopically. Macroscopic evaluation was performed on semen volume, pH, consistency, and color. Microscopic evaluation was performed on motility, viability, plasma membrane integrity (PMI), and sperm concentration.

Sperm motility was evaluated by placing 10 µl of semen and diluent on a glass slide and covering it with a cover glass. Motility was assessed as sperm moving progressively and was determined subjectively in 4–5 fields of view (Robeck & O'Brien 2004;

Yuen et al., 2009). The values given range from 0% to 100% with a scale of 5%. Sperm viability was evaluated using eosin-nigrosin staining. A total of 5 µl of semen was added to 20 µl of eosin-nigrosin. The two solutions were homogenized and smear preparations were made and dried at 37 °C. Observations were made using a microscope with 400× magnification, with a minimum of 200 sperm cells. Sperm PMI testing was performed using a hypoosmotic swelling test (HOST) according to the procedure described by Pardede et al. (2022).

Five microliters of semen were placed in a microtube containing 200 microliters of hypoosmotic solution. The sample was then incubated in a water bath at 37 °C for 30 minutes. Sperm that reacted and did not react with the hypoosmotic solution were counted in 10 fields of view with a total of 200 cells. Sperm with intact plasma membranes were marked with coiled tails, while those that were not intact had straight tails.

Liquid Semen Processing and Evaluations

Fresh semen showing sperm motility >70% was divided into four treatments with the addition of commercial lecithin-based diluents, each treatment differentiated by the presence or absence of centrifugation. The composition of the soy lecithin-based commercial diluent used in this study was as follows: tris, citric acid, phospholipids, fructose, glucose, antioxidants, pH buffers, glycine, soy lecithin: 1 g/L; glycerol: 38.47 g/L; antibiotics (Tylosin: 0.57 g/L, Gentamicin: 0.286 g/L, Spectinomycin: 0.343 g/L, Lincomycin: 0.172 g/L). Commercial lecithin-based diluents were diluted with aquabidest at a ratio of 1:4 according to the procedure on the packaging. The diluents were homogenized and stored at 21 °C until dilution.

The uncentrifuged semen sample was divided into two tubes, each diluted to a sperm concentration of 100 × 106 ml-1 and 200 × 106 ml-1 with a final volume after dilution of 2 mL. The centrifuged semen sample was centrifuged at 3000 rpm for 15 minutes, then the pellet was taken and the concentration was recalculated. After that, the sample was stored in two tubes and diluted according to the previous treatment. The semen samples were then stored at 5 °C for 4 days. The diluted and stored semen was evaluated every 24 hours for 4 days. The evaluation was performed microscopically according to the microscopic evaluation procedure previously performed on fresh semen.

Data Analysis

This study used a completely randomized design (CRD) for each stage with three replicates each. Data were tabulated using Microsoft Excel 36 and presented in the form of mean ± standard error of mean (SEM). Data obtained from each stage of the study were then analyzed statistically using Analysis of Variance (ANOVA) at a 95% significance level. If there were significant differences between treatments, Duncan's Multiple Range Test (DMRT) was used for further analysis.

RESULTS AND DISCUSSION

Fresh Semen Characteristics

The fresh semen characteristics, including volume, colour, consistency, acidity, motility, viability, abnormalities, concentration, and plasma membrane integrity of Indo-Pacific bottlenose dolphins, can be seen in Table 1. The quality of fresh semen shows that Indo-Pacific bottlenose dolphins are suitable for processing into liquid semen because they have fresh semen motility ≥70%. Good semen quality is influenced by a good maintenance system and a collection process that is carried out after undergoing regular training. The frequency of semen collection also affects the success of collection and the quality of semen produced (Takenaka et al., 2013).

Liquid Semen Characteristics

Sperm motility in liquid semen stored at 5 °C decreased daily, consistent with previous studies (Ruiz-Díaz et al., 2020). On day 4, sperm motility was at its lowest point during the evaluation and differed significantly ($P < 0.05$) from other days. Sperm motility in liquid semen on day 4 ranged from 11.67% to 20.00% in both males (Figure 1). The decline in sperm motility may be influenced by storage time and temperature, diluent type, and sperm concentration (Ruiz-Díaz et al., 2020). High sperm concentration can cause a decrease in motility due to high collision activity between sperm, with the probability increasing as sperm concentration increases (Van der Horst et al., 2018). High sperm concentration can also cause high metabolic output, associated with oxidative stress and the formation of reactive oxygen species (ROS),

Table 1 Fresh semen characteristics of Indo-pacific bottlenose dolphins

Parameter	Dolphin 1 (n=3)	Dolphin 2 (n=3)
Volume (mL)	24,67 ± 8,18	12,33 ± 6,06
Color	Milky white	Milky white
Consistenccy	Medium	Medium
pH	7,47 ± 0,21	7,57 ± 0,09
Sperm Motility (%)	86,67 ± 2,36	83,33 ± 2,36
Sperm Viability (%)	93,50 ± 0,97	92,42 ± 3,04
Sperm Abnormalities (%)	2,56 ± 0,59	3,12 ± 1,19
Sperm Consentration (× 10^6/mL)	758,33 ± 177,83	848,33 ± 614,96
Sperm PMI (%)	65,58 ± 3,81	66 ± 0,89

Data are presented as mean ± SEM

affecting the decline in sperm motility due to oxidative damage (Agarwal et al., 2014; Khoi et al., 2021).

Sperm motility values can be maintained at ≥40% up to 48 hours after collection with the addition of a diluent. The diluent contains tris, which acts as a pH regulator, preventing changes in hydroxide and hydronium ions, protecting sperm cells from pH changes during storage that can reduce sperm motility (Liu et al., 2016; Namula et al., 2019).

The diluent also contains phospholipids that play a role in maintaining pH levels and converting proteins, fats, and sugars into carbon dioxide. Phospholipids also play a role in sperm freezing and thawing, which is related to sperm motility (Tanni et al., 2022). In addition, the diluent contains lecithin, which protects the plasma membrane of cells from temperature changes during storage (Emamverdi et al., 2013).

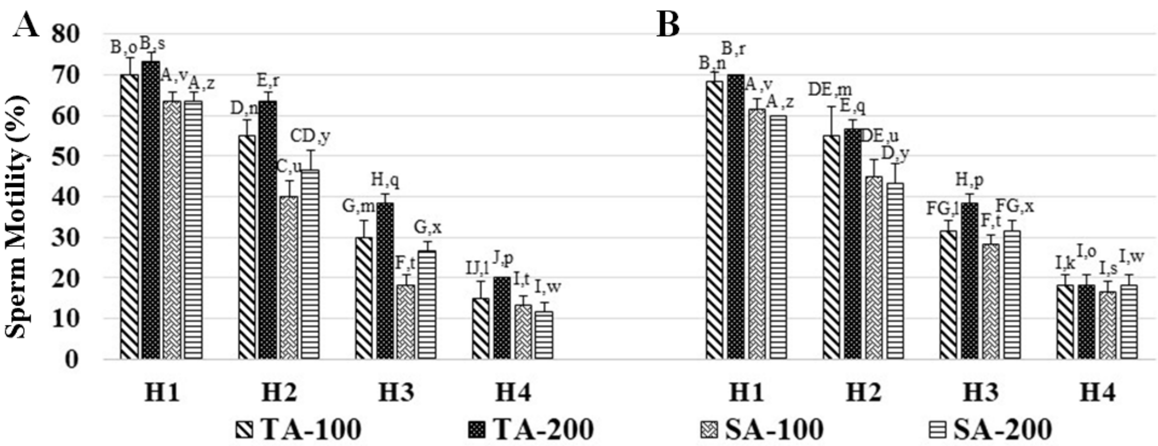

Figure 1. Sperm motility (A. Male 1; B. Male 2). Letters A-J indicate significant differences on the same day with different treatments ($P < 0.05$), letters k-z indicate significant differences on different days with the same treatment ($P < 0.05$); TA-100 (Semen and commercial lecithin-based diluent with a concentration of 100 × 106/mL without centrifugation), TA-200 (Semen and commercial lecithin-based diluent with a concentration of 200 × 106/mL without centrifugation), SA-100 (semen and commercial lecithin-based diluent with a concentration of 100 × 106/mL centrifuged), SA-200 (semen and commercial lecithin-based diluent with a concentration of 200 × 106/mL centrifuged)

The results showed that the motility on days 1 and 2 of the centrifugation treatment were significantly lower than those of the non-centrifugation treatment in Male 1 ($P < 0.05$). In the centrifugation treatment, the supernatant containing semen plasma was removed or remained in small amounts, which was thought to affect motility values. Semen plasma is positively correlated with sperm motility values and has a beneficial effect on liquid semen preservation (Takenaka et al., 2013). Semen plasma can act as a buffer and medium for sperm motility (Agarwal et al., 2014). The interaction between semen plasma and sperm is species-specific (Höfner et al., 2020).

In dogs, a diluent with an appropriate semen plasma concentration (25% semen plasma) was able to maintain the motility of liquid semen sperm for 96 hours (Pan et al., 2018). In sheep, semen plasma at concentrations of 20% and 40% had a protective effect on the motility of liquid semen sperm after 24 hours of storage (Mata-Campuzano et al., 2015). Additionally, in horses, the complete or partial removal of semen plasma by centrifugation for liquid semen can increase sperm motility during 48 hours of storage (Brinsko et al., 2000).

Sperm viability decreased over time during storage. On day 4, all treatments showed the lowest viability values with no significant differences between treatments. Sperm viability in both treatments ranged from 16.38% to 84.15% in both males (Figure 2). Viability values were maintained at >40% in all non-centrifuged treatments until day 3 after collection. In a study by Takenaka et al. (2013), no difference was found between centrifuged and non-centrifuged fresh semen in terms of sperm viability. However, it was negatively correlated with sperm concentration.

Higher sperm concentrations cause higher production of reactive oxygen species (ROS) from sperm metabolism, which can cause cell damage. However, another study reported that higher concentrations did not significantly affect viability in concentration comparisons (Ruiz-Díaz et al., 2020). This is consistent with the results of studies conducted with maximum concentrations of 200×10^6 ml^{-1}.

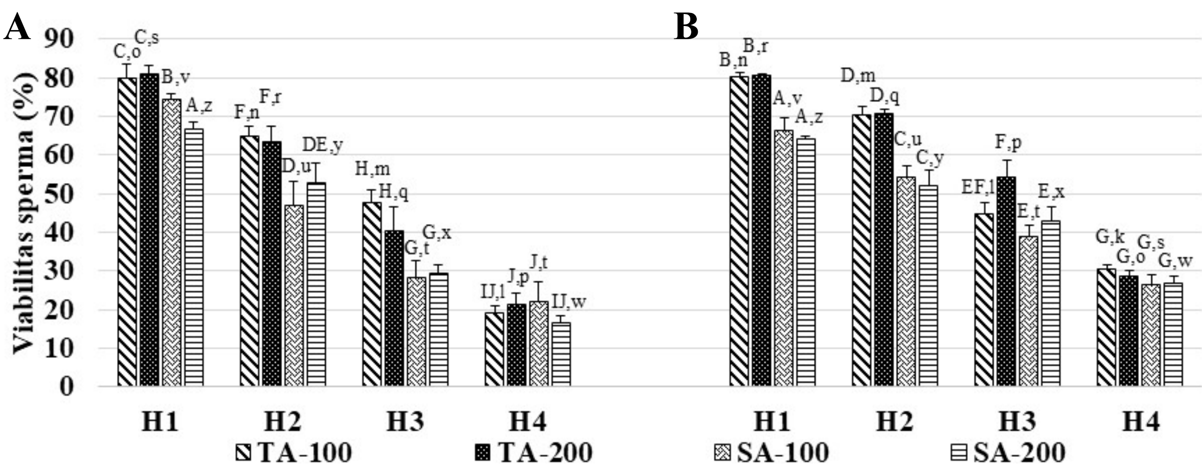

Figure 2. Sperm viability (A. Male 1; B. Male 2). Letters A-J indicate significant differences on the same day with different treatments ($P < 0.05$), letters k-z indicate significant differences on different days with the same treatment ($P < 0.05$); TA-100 (Semen and commercial lecithin-based diluent with a concentration of 100×10^6/mL without centrifugation), TA-200 (Semen and commercial lecithin-based diluent with a concentration of 200×10^6/mL without centrifugation), SA-100 (semen and commercial lecithin-based diluent with a concentration of 100×10^6/mL centrifuged), SA-200 (semen and commercial lecithin-based diluent with a concentration of 200×10^6/mL centrifuged)

The integrity of the sperm plasma membrane showed an interaction with storage time in all treatments (P < 0.05). Plasma membrane integrity values decreased with storage duration. On day 4, plasma membrane integrity values were at their lowest, ranging from 15.69 to 28.70% (Figure 3). Plasma membrane integrity is very important in protecting the inside of the cell. Temperature changes cause cell membrane damage during storage. Liquid semen preservation techniques can minimize extreme temperature changes. The addition of a diluting medium is also necessary to minimize the impact of storage temperature changes. Commercial diluents based on soy lecithin work by forming a protective layer on the surface of sperm cells (Emamverdi et al., 2013). Ready-to-use commercial lecithin diluents are available with the addition of distilled water, as the diluents already contain buffers with glycerol and antibiotics. Other diluents, such as commercial diluents, require the addition of distilled water and egg yolk as a source of lecithin in their preparation. The addition of egg yolk can cause inconsistencies in the results, such as in the preparation of homemade diluents, especially in the evaluation of plasma membrane integrity (Tarig et al., 2017).

The quality of liquid semen preservation yields better results at 5 °C storage temperature and a concentration of 100 × 10^6 sperm/ml in bottlenose dolphins (Ruiz-Díaz et al., 2020). In this study, the concentration values did not show significant differences between 100 x 10^6 sperm/ml and 200 x 10^6 sperm/ml for each treatment. In sheep, liquid semen preservation quality is good at concentrations below 0.8 × 10^9 sperm/ml (Mata-Campuzano et al., 2015). Meanwhile, reducing the sperm concentration below 500 × 10^6 sperm/mL does not improve sperm quality in goats (Sadeghi et al., 2020). Sperm concentration is an important variable that needs to be considered during the liquid semen preservation process. Suboptimal sperm concentration has been reported to be associated with changes in sperm structure and function variables as well as sperm motility in rams (Mata-Campuzano et al., 2015).

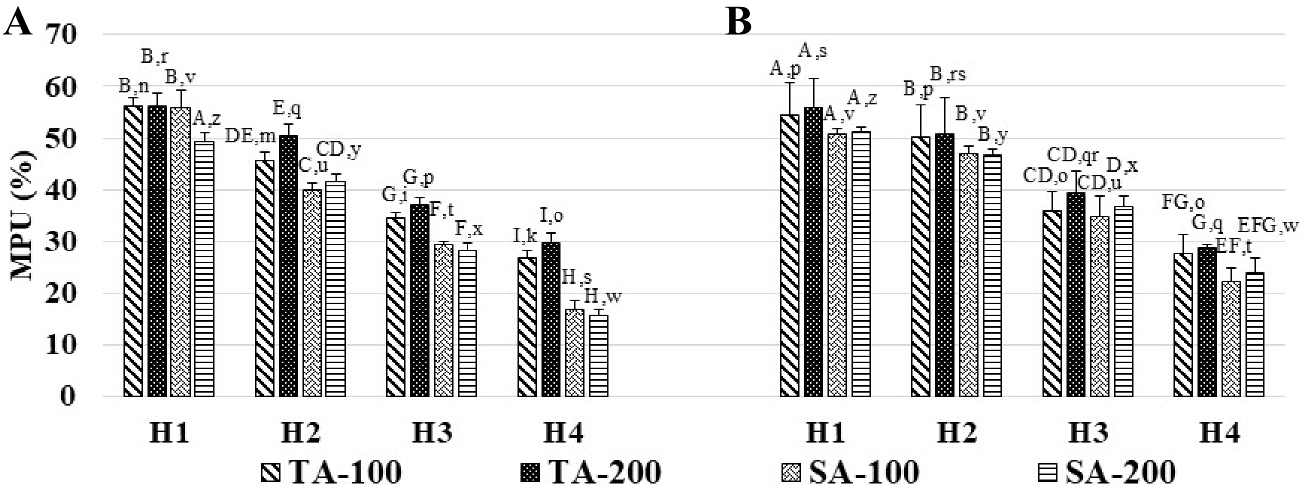

Figure 3. Integrity values of sperm plasma membranes (A. Male 1; B. Male 2). Letters A-I indicate significant differences on the same day with different treatments (P < 0.05), letters k-z indicate significant differences on different days with the same treatment (P < 0.05); TA-100 (Semen and commercial lecithin-based diluent with a concentration of 100 × 106/mL without centrifugation), TA-200 (Semen and commercial lecithin-based diluent with a concentration of 200 × 106/mL without centrifugation), SA-100 (semen and commercial lecithin-based diluent with a concentration of 100 × 106/mL centrifuged), SA-200 (semen and commercial lecithin-based diluent with a concentration of 200 × 106/mL centrifuged)

Liquid semen preservation can maintain sperm quality in all treatments until day 2. The decline is significant every day (P < 0.05). The decline occurred in the evaluation of motility, viability, and MPU. Semen evaluation could only be performed on two animals. Indo-Pacific bottlenose dolphins that have been trained and from which semen can be collected are only available at the WSI conservation institution.

Liquid semen preservation for Indo-Pacific bottlenose dolphin sperm can be carried out for a short storage period of 48 hours in all treatments using commercial lecithin-based diluents.

Further research is needed in the development of alternative diluents for the preservation of Indo-Pacific bottlenose dolphin semen for use in future artificial insemination programs.

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