VOLUME 13 NOMOR 1 JUNE 2026 ISSN 2548 – 611X

JURNAL
BIOTEKNOLOGI & BIOSAINS INDONESIA

Homepage Jurnal: http://ejournal.brin.go.id/JBBI/index

IMPROVING SEMEN QUALITY OF PERANAKAN ONGOLE CATTLE WITH MARIGOLD®-BASED ANTIOXIDANT IN TRIS-EGG YOLK DILUENT

Peningkatan Kualitas Semen Sapi Ongole dengan Antioksidan Berbasis Marigold® Pada Pengencer Tris-kuning Telur

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ABSTRACT

This study aimed to determine the optimal concentration of Marigold® antioxidant suplemented in a Tris-egg yolk (TEY) extender to maintain the quality of liquid semen from Peranakan Ongole (PO) bulls. Semen samples were extended in TEY without antioxidant (P0, control) or supplemented with Marigold® extract at concentrations of 0.002% (P1), 0.004% (P2), and 0.006% (P3). Semen quality was evaluated based on macroscopic characteristics (volume, colour, pH, and consistency) and microscopic parameters (mass motility, progressive motility, viability, plasma membrane integrity (PMI), and morphological abnormalities). Fresh semen exhibited an average volume of 5.25±0.8 mL, creamy colour, pH 6.8±0.13, mass motility (++), abnormalities 4.1±0.8%, viability 86.12±6.2%, PMI 77.21±5.3%, and progressive motility 85.44±2.41%. Supplementation with Marigold® antioxidant significantly influenced microscopic parameters during storage at 4 °C. Sperm abnormalities increased with storage duration, particularly in P3 on day 2. Treatment with 0.004% Marigold® (P2) effectively maintained motility and PMI up to day 6, while 0.006% (P3) preserved viability and PMI up to day 10. The addition of Marigold® extract to the TEY extender improved the preservation of PO bull semen quality during 10 days of storage at 4 °C. A concentration of 0.004% was optimal for maintaining sperm quality in the short term (up to day 6), whereas 0.006% provided stronger protective effects during prolonged storage (up to day 10).

Keywords: *Marigold® antioxidant, Local cattle, Ongole crossbreed (PO) cattle*

ABSTRAK

Penelitian ini bertujuan untuk mengetahui konsentrasi optimal antioksidan Marigold® dalam pengencer Tris-kuning telur (TKT) untuk mempertahankan kualitas semen cair sapi Peranakan Ongole (PO). Semen diencerkan dengan pengencer TKT (P0) yang ditambahkan ekstrak Marigold® 0,002% (P1), 0,004% (P2), dan 0,006% (P3). Kualitas semen dievaluasi secara makroskopis (volume, warna, pH, konsistensi) dan mikroskopis (gerakan massa, motilitas, viabilitas, MPU, abnormalitas). Kualitas semen segar menunjukkan rata-rata volume 5,25±0,8 ml, warna krem, pH 6,8±0,13, gerakan massa (++), abnormalitas 4,1±0,8%, viabilitas 86,12±6,2%,

Received: 01 August 2025 *Accepted*: 05 April 2026 *Published*: 09 June 2026

MPU 77,21±5,3%, serta motilitas progresif 85,44±2,41%. Penambahan ekstrak Marigold® memengaruhi parameter mikroskopis selama penyimpanan pada suhu 0-4°C. Abnormalitas meningkat seiring waktu, terutama pada P3 hari ke-2. P2 mempertahankan motilitas dan MPU hingga hari ke-6, sedangkan P3 menjaga viabilitas dan MPU hingga hari ke-10. Kesimpulannya penelitian ini adalah penambahan antioksidan Marigold® ke dalam pengencer TKT dapat mempertahankan kualitas semen dan berpengaruh positif terhadap kualitas semen cair sapi PO selama masa penyimpanan 10 hari suhu 4ºC. Perlakuan 0,004% efektif mempertahankan kualitas spermatozoa hingga hari ke-6, sedangkan perlakuan 0,006% memberikan efek protektif dalam jangka panjang (hingga hari ke-10).

Kata kunci: *Antioksidan marigold®, Sapi lokal, Sapi Peranakan Onggole (PO)*

INTRODUCTION

Ensuring an adequate supply of animal protein remains a national priority in Indonesia, as demonstrated by the government's beef self-sufficiency program targeted for 2026. However, rapid population growth, reaching 281.6 million people in 2024 (BPS, 2024), has outpaced improvements in both the quantity and quality of domestic cattle production. One strategy approach to support this program is the genetic improvement and sustainable utilization of indigenous cattle resources, particularly Peranakan Ongole (PO) cattle. PO cattle are descendants of the Ongole breed originally imported from India in the early 20th century and have undergone long-term adaptation and genetic stabilization under Indonesian tropical conditions, especially on Sumba Island. This breed is well recognized for its high adaptability to harsh tropical environments, tolerance to low-quality feed resources, and resistance to parasites (Khoirunnisa and Utomo, 2024).

Artificial insemination (AI) has been widely implemented to enhance beef cattle productivity; however, its success is highly dependent on semen quality, technical proficiency, and the effectiveness of semen preservation methods (Mulu et al., 2018). Semen extenders play a crucial role in maintaining spermatozoa viability and motility during storage. Among various extenders, Tris-based formulations are extensively used due to their strong buffering capacity and ability to preserve sperm motility during cold storage (Arif et al., 2022). Despite these advantages, semen preservation commonly results in a 10-40% reduction in semen quality, primarily due to oxidative stress caused by excessive production of reactive oxygen species (ROS) during cooling and storage (Baharun et al., 2017). Elevated ROS levels can damage sperm plasma membranes, impair mitochondrial function, reduce motility, and ultimately compromise fertilization capacity (Qamar et al., 2023; Wang et al., 2025).

To mitigate oxidative stress during semen preservation, supplementation of semen extenders with natural antioxidants has received increasing attention. Several plant-derived antioxidants, including green tea, moringa leaf, and turmeric extracts, have been reported to improve sperm motility, viability, and membrane integrity in cattle and other livestock species (Authaida et al., 2025). Nevertheless, research on the application of Marigold® (*Tagetes erecta*) extract in semen preservation remains limited and is largely restricted to non-ruminant species. Marigold® extract is rich in bioactive compounds such as flavonoids and carotenoids, which possess strong antioxidant properties. Moreover, marigold is widely cultivated, relatively inexpensive, and readily available in tropical regions, making it a potentially sustainable antioxidant source for livestock applications. The lack of studies evaluating its effects on ruminant semen, particularly in indigenous cattle breeds, represents a significant knowledge gap.

Therefore, this study aimed to evaluate the effects of different concentrations of Marigold® (*Tagetes erecta*) extract supplementation in a Tris-egg yolk (TEY) extender on the quality of PO cattle semen during liquid storage at 4°C. By determining the optimal concentration of Marigold® antioxidant, this research introduces a novel approach to improving semen preservation with direct practical implications for AI programs. The application of Marigold®-supplemented extenders not only offers a natural and cost-effective antioxidant alternative but also contributes to the conservation and sustainable utilization of PO cattle as part of Indonesia's beef self-sufficiency strategy.

MATERIALS AND METHODS

Experimental Animals

Two selected PO bulls, approximately 5 years of age, were used as semen sources. The bulls were housed individually under standardized management conditions in compliance with established animal welfare standards. All animals were fed fresh forage/grass at 10% of body weight and concentrate at 1% of body weight, with drinking water provided *ad libitum*.

Extender Preparation

The TEY extender was prepared using a buffer solution consisting of 2.472 g Tris (hydroxymethyl) aminomethane, 1.384 g citric acid, and 1.016 g fructose dissolved in distilled water to a final volume of 100 mL. The solution was homogenized throughly and subsequently placed in a water bath, heated indirectly for 10 minutes at 92-95 °C, and then allowed to cool gradually to room temperature. After cooling, antibiotics were added under continuous stirring, followed by the addition of fresh egg yolk, and mixed thoroughly to ensure homogeneity. Ejaculates that met the minimum quality standards were diluted with the prepared TEY extender for further evaluation.

Semen Collection and Evaluation

Semen was collected from PO cattle using an artificial vagina in the morning, with a collection frequency of twice per week. Fresh ejaculates were immediately evaluated both macroscopically and microscopically. Macroscopic assessments included semen volume, colour, mass motility, and pH (using special indicator paper, 6.4-8.0: Merck®).

Progressive sperm motility was measured using computer-assisted sperm analysis (CASA) instrument Androvision® (Minitub-Germany). Sperm concentration was determined with a photometer (SDM-6, Minitüb®, Germany). Sperm viability and morphological abnormalities were assessed using eosin-nigrosin staining. Plasma membrane integrity was evaluated by mixing 10 µL of semen with 1 µL of hypo-osmotic swelling (HOS) solution (prepared with 7.35 g sodium citrate and 13.52 g fructose in 1000 mL of distilled water).

Diluted semen samples were suplemented with Marigold® antioxidant according to the following treatments: P0: TEY extender without Marigold® (control); P1: TEY extender + 0.002% Marigold®; P2: TEY extender + 0.004% Marigold®; and P3: TEY extender + 0.006% Marigold®. All extended semen samples were stored at 4°C and evaluated for progressive motility, sperm kinematic parameters (CASA), and morphological abnormalities for up to 10 days of storage.

Data Analysis

Data were analyzed using analysis of variance (ANOVA) under a Completely Randomized Design (CRD). When significant differences ($p<0.05$) were detected, mean comparison were performed using Duncan's Multiple Range Test (DMRT). All analyses were conducted with SPSS software version 23 (IBM Corp., Armonk, NY, USA). Results are presented as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Fresh semen from PO Cattle

The evaluation of fresh semen from PO cattle is presented in Table 1.

Table 1. Evaluation of fresh semen from Ongole crossbred (PO) cattle

Parameters	Average±SD
Macroscopic	
Volume	5.25±0.80
Consistency	Medium-thick
pH	6.8±0.13

Parameters	Average±SD
Colour	Cream
Microscopic	
Mass movement	++
Progressive motility (%)	85.44±2.41
Motility (%)	89.19±4.40
Concentration (x 10^6)	1666±0.30
Viability (%)	86.12±6.20
PMI (%)	77.21±5.30
Abnormality (%)	4.1±0.80
VAP (µm/s)	101.95±5.88
VCL (µm/s)	174.12±6.54
VSL (µm/s)	71.30±1.54
STR (%)	0.69±0.03
ALH (µm/s)	6.07±0.50
BCF (%)	27.76±0.87

PMI (plasma membrane integrity), VAP (average path velocity), VCL (curve linier velocity), VSL (straight line velocity), STR (straightness), ALH (amplitudo if lateral head movement), BCF (beat cros frequency).

Assessment of fresh semen is essential to determine semen quality prior to processing into frozen semen suitable for AI. The results showed that, macroscopically, PO bull semen had an average volume of 5.25±0.8 mL, cream colour, medium to thick consistency, and an acidity level (pH) ranging from 6.0 to 7.0. Microscopic examination showed ++ mass motility and 85.44±2.41% progressive motility. The sperm concentration was 1666±0.3×10^6 mL^{-1}, with sperm viability of 86.12±6.2**%**. The percentage of sperm with intact plasma membranes was 77.21±5.3%, while sperm abnormality were recorded at 4.1±0.8%.

The observed macroscopic semen characteristics are include volume, colour, pH, and consistency. The semen volume of 5.25±0.8 mL obtained in this study aligns with previous findings that reported PO cattle semen volume ranging from 5.3 to 6.6 mL, with a whitish-yellow colour and medium to thick consistency (Nugraha and Pangestu 2023). Semen volume represents the total ejaculate released in one ejaculation and normally ranges from 4 to 8 mL (Mangun et al. 2025*)*. Semen consistency is related to sperm density, where thicker semen indicates a higher concentration of sperm in Pasundan bulls (Baharun et al. 2025).

The acidity level (pH) of semen in this study was 6.8±0.13, consistent with previous studies reporting that fresh PO cattle semen has a pH range of 6.3-7.0 *(Sukirman* et al. 2019). Semen pH is a critical indicator of sperm metabolic activity, where low pH may indicate the accumulation of lactic acid from sperm metabolic activity (Magun et al. 2025). Comparable semen characteristics have been reported in Pasundan bulls, with semen pH of 6.4 and medium-thick consistency (Baharun et al. 2023).

Higher mass motility indicates a greater likelihood of successful fertilization (Said et al. 2025). In this study, the mass motility of sperm was classified as good (++). The normal range of sperm mass motility is categorized as good (++) to very good (+++). These findings are consistent with Sukirman et al. (2019), who reported that PO bull sperm with mass motility scores of ++ to +++ and demonstrating progressive movement in dense cloud-like formations are considered to be within the normal category and are suitable for further processing in the AI.

The average sperm concentration of fresh semen from PO cattle in this study was 1666±0.30x10^6 mL^{-1}. Previous researchers reported that the average sperm concentration of PO bull was 1360±0.16 x10^6 mL^{-1} and 1480±302.43 x10^6 mL^{-1} (Sukirman et al. 2021; Nugraha and Pangestu 2023). Variation in bull sperm concentration are influenced by genetic quality, scrotal volume, and semen collection frequency (Murphy et al. 2018; Wijayanti et al. 2023). The viability of fresh semen in this study was 77.21±5.3%. This value aligns with several previous studies that showed viability values for fresh semen from PO bull ranged from 71.82% to 83.66% (Nugraha et al. 2022; Yuniar et al. 2024).

The PMI test in this study yielded a value of 89.19±4.4%. This is consistent with previous research reports that found the PMI of PO bull was 85.76±4.57, meeting the requirements for freezing (Mangun et al. 2025). The intact plasma membrane results of PO cattle in this study were higher than the intact plasma membrane of Bali bull (72.00±2.71%) and Pasundan bull (74.43±1.34%), the difference in PMI values was due to differences in breeds in bull (Iskandar et al. 2022; Baharun et al. 2023).

The individual motility value of fresh semen from PO cattle in this study was 89.19±4.40%. This value falls within the normal range of fresh semen motility, which is 70-90%. Sperm motility below 40% generally indicates poor semen quality, as fertile sperm typically exhibit progressive active motility within the range of 50-80% (Mangun et al. 2025).

Sperm abnormalities are an indicator for assessing abnormalities in sperm morphology. High levels of abnormalities can potentially lead to infertility (Baharun *et al*. 2021). The percentage of sperm abnormalities in this study was 4.1%, below the required abnormality rate. Previous reports stated that abnormalities in fresh semen from PO bull ranged from 0.50 to 3.50% (Sukirman et al. 2019). The percentage of frozen semen abnormalities below 20% is in accordance with Indonesian National Standard (SNI number: 4869.1:2024 for frozen semen bovine semen) frozen semen twhich is still suitable for insemination (BSN 2024).

Based on the research results, the average progressive motility value was 85.44±2.41%. The sperm motility results were suitable for further frozen semen processing because they were ≥70%. Motility is a very important factor for sperm to pass through the cervix. In fact, progressive motility helps sperm penetrate the cumulus oophorus and zona pellucida ovum, thus enabling fertilization (Kudratullah et al. 2025).

The kinematic parameters were evaluated using a Computer Assisted Sperm Analysis (CASA) system (Vision Version™ 3.7.5, Minitube, Germany), including distance curve linear (DCL), distance average path (DAP), distance straight line (DSL), velocity curve linear (VCL), velocity average path (VAP), velocity straight line (VSL), linearity (LIN), straightness (STR), and wobble (WOB). (Maulana et al. 2022). The average values of VAP, VCL, and VSL in this study were 101.95±5.88, 174.12±6.54, and 71.30±1.54, respectively. Velocity is the speed of sperm on a curved path (VCL), on a straight path (VSL), and on the average path of its flow (VAP) (Diansyah *et al.* 2022). The VCL, VSL, and VAP values of fresh Balinese bull semen were 133.62±10.08, 70.03±4.85, and VSL 45.50±3.6, respectively (Diansyah et al. 2022). Reported VCL, VSL, and VAP values in Ongole bull semen in previous studies were 105.30±12.15, 65.00±10.01, 53.00±11.84 (Sarastina et al. 2020). VAP values greater than 25 μm/s, with progressive movement speeds exceeding 20 μm/s, can significantly impact fertilization success (Santoso et al. 2024).

The average STR of fresh semen from PO cattle in this study was 0.69±0.03. STR is an indicator of progressive motility and movement patterns. Previous studies reported that the STR of Ongole bull was 0.81±0.08, Bali bull 0.82±0.07, and Madura bull 0.85±0.05 (Sarastina et al. 2020). Sperm with an average STR value greater than 50% are capable of moving forward in a linear trajectory (Santoso et al. 2024). The average ALH and BCF values in this study were 6.07±0.50 and 27.76±0.87, respectively. Previous studies reported that Ongole bull had an ALH value of 4.02±0.77 and BCF of 28.91±6.83 (Sarastina et al. 2020). In Bali bull, ALH was 6.54±0.21 and BCF was 31.49±0.63. An ALH value above 7 indicates hyperactive sperm (Diansyah et al. 2022). ALH is the degree of displacement or deviation of the sperm head during its movement and BCF is the frequency of sperm movement per minute (Santoso et al. 2024).

Evaluation of PO Bull Liquid Semen

Microscopic evaluation of PO cattle liquid semen sperm with a comparison of Tris Egg Yolk control diluent (20%) and the addition of marigold antioxidants is presented in Table 2. The amount of antioxidants needed from marigold flower extract with different concentrations aims to reduce the occurrence of ROS. The addition of Marigold® to liquid semen of PO cattle had a significant effect ($P<0.05$) on the motility and viability of individual sperm, but had no significant effect ($P>0.05$) on PMI and sperm abnormalities.

Table 2. Evaluation of sperm in Tris egg yolk-marigold® diluent with the addition of various concentrations

Parameterers	Control (Average±SD)	Marigold® (Average±SD)		
	P0	P1	P2	P3
Motility (%)	77.37±7.22^{a}	86.92±0.88^{a}	87.03±0.79^{ab}	81.73±0.99^{b}
Viability (%)	95.38±2.13^{a}	89.36±0.98^{b}	87.72±1.37^{b}	88.58±1.55^{b}
PMI (%)	92.00±4.44^{a}	88.27±1.04^{a}	89.33±1.51^{a}	89.57±2.79^{a}
Abnormality (%)	5.66±2.60^{a}	5.96±0.58^{a}	4.39±0.47^{a}	5.11±0.82^{a}

$^{a.b}$ Different superscripts in the same row indicate significant differences ($P<0.05$); P1= addition of marigold® flower extract of 0.002%; P2 = 0.004%; and P3 = 0.006%.

Sperm motility increased in treatments P1 (86.92%) and P2 (87.03%) compared to the control (77.37%). Treatment P2 was still not significantly different from the control, but P3 (81.73%) was significantly different from P2. The results of this study indicate that certain concentrations of Marigold extract can increase sperm motility, but this increase is not always linear. Motility serves as a primary indicator of sperm quality and its potential for successful fertilization (Baharun et al. 2023). The viability of sperm in liquid semen of PO bull with a comparison of the control diluent TEY (20%) and the addition of a percentage of Marigold® extract in this study showed a significant decrease compared to the control ($P<0.05$). This decrease in viability is suspected to be due to the toxic properties of Marigold extract (Merdana et al. 2024). Marigolds contain flavonoids, phenols, alkaloids, and tannins. Previous research suggests that flavonoids play a crucial role in reducing the effects of ROS-induced cell damage. Flavonoids bind to ROS by donating an H^{+} ion. Phenols can also be toxic and cause sperm death when used in high doses (Wajdi et al. 2021).

The results of this study indicate that the addition of Marigold® to TEY diluent maintains the stability of the sperm plasma membrane structure. TEY contains various important components such as Tris (hydroxymethyl aminomethane), citric acid, and fructose. The addition of antioxidants to the diluent has significant benefits, including preventing free radical activity that damages sperm cell membranes, which can impact sperm fertility (Wijayanti et al. 2023).

Sperm abnormalities in the liquid semen of PO cattle with a comparison of the TEY control diluent (20%) and the addition of various concentrations of Marigold extract showed relatively similar average values ($P>0.05$). The abnormality values in this study ranged from 4.39 to 5.96, which are still within the acceptable standard of less than 20%, indicating that the semen remains suitable for further treatment (Wijayanti et al. 2023). Baharun et al. (2023) reported that post-thaw Limousin sperm exhibited the lowest abnormality and highest normality values at 37 °C compared to lower thawing temperatures, thereby supporting the recommendation of this temperature as an optimal thawing protocol. Conversely, lower thawing temperatures were associated with increased sperm abnormalities and a reduction in the proportion of morphologically normal sperm.

The addition of Marigold® extract can maintain sperm motility, but optimization of the Marigold extract dose is still needed to obtain maximum benefits without reducing other important parameters. Benko et al. (2019) Marigold flower extract has been shown to improve sperm quality in Friesian Holstein bulls, including increasing mitochondrial motility and activity, and protecting sperm from damage caused by oxidative stress. Marigold extract has the potential to be used as an antioxidant protector to improve the quality of frozen semen.

Evaluation of sperm longevity in TEY-Marigold® diluent

Evaluation of sperm longevity in TEY diluent with the addition of Marigold® at different concentrations in a temperature deviation of 4 ºC in a refrigerator with observations for 6 times at 2 days (H) consisting of observations of motility, viability, PMI and abnormality parameters (Table 3). Sperm longevity is the ability of sperm to survive at a certain temperature (Sulistya et al. 2015). Based on the analysis of variance, it shows that the storage period has an effect sperm motility, viability, PMI and abnormalities significantly (P<0.05).

Table 3. Evaluation of longevity of liquid semen of PO cattle

Long storage days	Treatments			
	P0	P1	P2	P3
Motility				
H0	77.37±7.22^{aA}	86.92±0.88^{aA}	87.03±0.79^{abA}	81.73±0.99^{bA}
H2	69.78±0.72^{aB}	67.81±1.02^{bB}	63.83±0.32^{cB}	62.22±1.39^{cB}
H4	59.82±0.92^{aC}	55.01±1.14^{aC}	58.84±2.03^{aC}	60.51±0.62^{bC}
H6	60.37±1.26^{aC}	55.84±0.90^{abC}	61.06±1.33^{bcC}	57.97±1.71^{cC}
H8	44.93±1.15^{aD}	42.15±2.36^{aD}	44.83±2.35^{aD}	41.71±3.66^{aD}
H10	40.21±1.86^{aE}	40.94±2.80^{aE}	41.81±1.88^{aE}	37.04±1.56^{aE}
Viability				
H0	95.38±2.13^{aA}	89.36±0.98^{bA}	87.72±1.37^{bA}	88.58±1.55^{bA}
H2	68.34±0.77^{aB}	65.29±0.48^{bB}	63.94±0.21^{cB}	60.24±0.92^{dB}
H4	60.43±1.55^{aC}	58.46±1.61a^{bC}	62.76±1.16^{bC}	59.78±1.45^{bC}
H6	47.24±2.86^{aD}	47.07±1.17^{bD}	52.08±2.14^{bD}	48.53±2.51^{bD}
H8	44.88±1.27^{aE}	39.86±1.35a^{bE}	42.84±2.72a^{bE}	47.29±4.92^{bE}
H10	38.28±2.24^{aF}	38.08±1.74a^{bF}	41.30±0.99^{bF}	42.73±1.89^{bF}
PMI				
H0	92.00±4.44^{aA}	88.27±1.04^{aA}	89.33±1.51^{aA}	89.57±2.79^{aA}
H2	77.02±0.76^{aB}	70.00±0.19^{aB}	75.97±1.07^{bB}	68.31±0.88^{cB}
H4	63.70±1.83^{aC}	67.93±2.14^{aC}	65.68±0.66^{aC}	64.57±0.67^{aC}
H6	63.76±2.09^{aD}	57.52±1.86^{bD}	59.13±1.72^{bcD}	54.99±2.33^{cD}
H8	50.49±1.18^{aE}	50.24±1.51^{abE}	52.31±2.13^{abE}	48.43±1.29^{bE}
H10	40.22±2.05^{aF}	45.72±1.51^{abF}	43.77±0.61^{bcF}	47.86±3.28^{cF}
Abnormality				
H0	5.66±2.60^{aA}	5.96±0.58^{aA}	4.39±0.47^{aA}	5.11±0.82^{aA}
H2	14.65±0.21^{aB}	16.61±0.44^{aB}	16.88±0.98^{bB}	21.33±0.20^{cB}
H4	20.21±1.00^{aC}	16.74±1.19^{aC}	20.65±1.34^{aC}	22.02±1.68^{bC}
H6	21.46±1.25^{aD}	22.59±1.04^{aD}	23.42±0.85^{aD}	23.16±1.60^{aD}
H8	26.16±1.22^{aE}	22.88±1.86^{abE}	24.64±1.87^{abE}	25.71±0.97^{bE}
H10	27.18±1.20^{aF}	25.31±2.88^{aF}	25.41±2.56^{aF}	28.82±1.85^{aF}

Different lowercase letters (^{a,b,c}) in the same row are significantly different (P<0.05); Different uppercase letters ($^{A, B, C, D, E, F}$) in the same column are significantly different (P<0.05); P1= Addition of marigold® extract of 0.002%; P2 = 0.004%; and P3 = 0.006%.

Marigold® is known to contain active compounds such as flavonoids, alkaloids, tannins, and phenols, which function as natural antioxidants. Antioxidants play a role in protecting sperm from damage caused by ROS, which increases during storage at 0-4ºC. Treatment effectiveness varies depending on the percentage and parameters observed. Treatment P2 showed optimal results in maintaining motility, viability, and PMI in days 0-6. Meanwhile, treatment P3 was superior in maintaining viability and PMI on days 8-10 and showed long-term effects even though motility was not very high on day 0, P1 and P2 produced significantly higher motility compared to the control

(P<0.05), indicating that Marigold® can increase the initial motility activity of sperm. P2 treatment maintained better motility until day 6. Storage days 8 and 10 showed no significant difference between treatments (P>0.05) indicating that the decrease in motility after day 6 is difficult to avoid even with antioxidant treatment from Marigold®. Marawali et al. (2019) reported that the motility of Bali bull sperm was able to survive 40% until the 3 days.

Viability showed a progressive decline during storage across all treatments (P<0.05). On day 0, the control (P0) had significantly higher viability compared to treatments with Marigold® (P1-P3) (P<0.05). On days 6 to 10, P3 tended to produce higher viability than the other treatments. This indicates that Marigold® treatment P3 is able to provide long-term protection against cell death, presumably due to the antioxidant content such as flavonoids and phenols that can neutralize free radicals during storage. Damaged sperm membranes are unable to provide enzymes in the metabolic process, so the availability of energy to maintain viability is reduced (Sun et al. 2020). The natural antioxidants contained in marigold have been shown to protect sperm from damage caused by free radicals (Benko et al. 2019).

The PMI of PO bull decreased gradually (P<0.05) during the observation of longevity. On days 2 to 8, P2 had the highest PMI value and was significantly different compared to other treatments (P<0.05). On day 10, the highest PMI occurred in treatment P3. This indicates that P2 is more effective in the early stages of storage, while P3 provides long-term protective effects by slowing membrane damage due to accumulated oxidative stress. The results of this study are in line with previous research by Iskandar et al. (2022) who reported that the Intact PMI of Bali bull sperm from the first to the fourth day of observation from all treatments of the percentage of sugarcane water extract diluent with the addition of egg yolk showed a fairly high average, which is above 60%. Furthermore, sperm viability declined from 87.47% to 77.27%, and motility dropped from 81.10% to 70.22% (p<0.05). PM and PMI also showed significant reductions. The low PMI is associated with sperm plasma membrane damage due to the generation of oxidative stress through the cGMP mechanism. Increased intracellular cGMP signals physiology responses through two cyclic pathways: the cGMP-protein kinase G (PKG), cGMP-regulated phosphodiesterases (PDE2, PDE3), and cGMP-gated cation channels mediated by PKG-substrate-specific activation of PKG1 result in decreased cytosolic calcium concentration and decreased sensitivity of myofilaments to Ca^{2+} desensitization (Baharun et al. 2023). Kinematic analysis showed a significant decrease in VCL, VSL, and VAP post-thaw. Additionally, parameters such as STR, LIN, and BCF were found to be considerably reduced (Diansyah et al. 2022).

Sperm abnormalities increased with increasing storage time in all treatments (P<0.05). On day 0, there was no significant difference between Marigold extract treatments, but day 2 showed that treatment P3 had the highest level of abnormalities. This indicates that excessively high doses of marigold extract (P3) have the potential to cause oxidative stress or osmotic imbalance and can trigger an increase in sperm morphological abnormalities. Based on the results of the analysis of variance test, treatment P1 provided relatively optimal protection against sperm structural damage until day 4. Marigold® extract has the potential as a protective agent for sperm morphology, but must be used in optimal concentrations. Research by Sumadiasa et al. (2022) reported that guava filtrate in egg yolk-citrate diluent was able to suppress sperm abnormalities far below the maximum standard (20%) for up to 7 days of storage. Sperm quality is largely determined by the structure of the sperm cells.

CONCLUSION

The addition of Marigold® antioxidants in the TEY extender at a concentration of 0.004% was effective in maintaining sperm quality until day 6. Meanwhile, the 0.006% treatment provided a stronger protective effect in the long term (up to day 10). The combination of Marigold® antioxidants with proper temperature management and storage techniques has the potential to increase the success of AI technology.

ACKNOWLEDGMENTS

This study was supported by the National Research and Innovation Agency (BRIN) and Indonesia Endowment Fund for Education Agency (LPDP) through Riset dan Inovasi untuk Indonesia Maju (RIIM) (Agreement Number: 114/IV/KS/11/2023 and 198/04/NK-X/XI/2023).

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