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Effect of Palmyra Fruit Water–Egg Yolk Diluent with Moringa Leaf Extract on Boar Sperm Membrane Integrity and Motility

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Abstract. This study aimed to evaluate the effect of palmyra (*Borassus flabellifer* L.) fruit water–egg yolk diluents supplemented with moringa (*Moringa oleifera* L.) leaf extract on the intact plasma membrane, intact acrosomal membrane, and motility of boar spermatozoa during liquid storage. A completely randomized design was used, with seven diluent formulations varying in concentrations of egg yolk and moringa leaf extract. Each treatment was replicated four times and stored at 15-18°C for up to 72 hours. Semen quality was evaluated at 0, 24, 48, and 72 hours of storage. Data were analyzed using analysis of variance followed by Duncan's multiple range test. Both diluent formulation and storage duration significantly influenced all semen quality parameters ($p < 0.05$). The diluent containing palmyra fruit water supplemented with 20% egg yolk and 15% moringa leaf extract produced the highest membrane integrity, motility, and viability with the lowest abnormality rate. The semen maintained acceptable quality for artificial insemination for up to 48 hours of storage. The superior performance of this formulation is biologically associated with the synergistic interaction between carbohydrate-derived energy from palmyra fruit water, phospholipid-mediated membrane stabilization from egg yolk, and antioxidant protection from moringa leaf extract against oxidative stress.

Keywords: Lontar fruit water; egg yolk; moringa leaf extract; intact plasma membrane (IPM); intact acrosomal membrane (IAM); progressive motility; viability; sperm abnormality.

I. INTRODUCTION

Bali represents one of Indonesia's major regions for pig production, where pig farming plays an important socio-cultural and economic role. Efforts to increase pig numbers and improve genetic quality require the implementation of effective reproductive technologies, including artificial insemination. Artificial insemination (AI) has been widely adopted to improve reproductive efficiency, genetic progress, and maximize the utilization of superior boars.

The success of AI implementation is largely determined by the quality of the semen used. One of the main challenges in AI implementation is maintaining semen quality so that it remains suitable for use over time, thereby ensuring a sustainable semen supply. To slow down sperm metabolism, fresh semen is usually kept as liquid semen in pigs at a cool temperature (15–18°C) [1]. However, liquid storage at low temperatures predisposes spermatozoa to cold shock and oxidative stress, primarily due to excessive production of reactive oxygen species (ROS), which can cause lipid peroxidation, decreased plasma membrane and acrosomal membrane integrity, DNA damage, and cell death [2-3].

The use of appropriate diluents is key to maintaining semen quality during storage. An ideal diluent should provide an energy source, maintain osmotic balance,

protect sperm membranes from damage caused by low temperatures, and contain protective components against oxidative stress. Palmyra fruit juice is a natural ingredient with potential use as an alternative diluent due to its relatively high carbohydrate content, particularly glucose, fructose, and sucrose [4]. These carbohydrates not only serve as an energy source for sperm but also act as extracellular cryoprotectants, reducing the impact of cold shock during semen storage [5-6].

In addition to an energy source, liquid semen diluents generally require membrane-protecting ingredients, such as egg yolk. Egg yolk contains phospholipids and cholesterol, which play an important role in stabilizing sperm membranes and protecting them from damage caused by temperature changes [4][7]. Cholesterol in egg yolks has been shown to increase sperm membrane resistance to cold shock, thereby maintaining viability and motility during storage [5][8-9].

On the other hand, oxidative stress caused by ROS during semen storage needs to be controlled by adding antioxidants. Moringa leaves (Moringa oleifera L.) are known to contain high levels of antioxidants, such as flavonoids, alkaloids, tannins, and other phenolic compounds [6]. The antioxidants in moringa leaf extract can neutralize free radicals and protect cell biomolecules from oxidative damage, potentially maintaining membrane integrity and the physiological function of spermatozoa during semen storage [7].

Although previous studies have independently evaluated the use of palmyra fruit water, egg yolk, or moringa leaf extract as semen diluent components, limited information is available regarding their combined synergistic effects in a single formulation for liquid-stored boar semen [8-10]. Furthermore, no study has specifically examined the optimal concentration balance between membrane stabilizers and natural antioxidants in palmyra-based diluents. Therefore, a clear research gap exists concerning the development of an integrated natural diluent formulation capable of simultaneously providing energy supply, membrane protection, and oxidative stress control during liquid storage. Therefore, this study aims to evaluate and determine the most effective palmyra fruit water–egg yolk diluent formulation supplemented with moringa leaf extract for maintaining boar sperm membrane integrity and motility during liquid storage.

II. METHODS

Research Ethics Statement

All research procedures have been reviewed and approved by the Experimental Animal Ethics Committee of the Faculty of Veterinary Medicine, Udayana University, with approval number 242/UN14.2.9/PT.01.04/2024.

Research Materials and Equipment

The materials used in this study included Landrace boar semen, palm fruit juice, broiler chicken eggs, Moringa leaves, sodium chloride, sodium citrate, fructose, eosin Y, nigrosin, 70% alcohol, streptomycin, crystalline penicillin, aquabidest, and other supporting materials. All chemical reagents used in this study were of analytical grade. The equipment used included a binocular microscope, Neubauer hemocytometer, Pasteur pipettes, volumetric pipettes, glass slides and coverslips, pH meter, test tubes, water bath, centrifuge, analytical balance, cool box, ice packs, and other supporting laboratory equipment.

Research Design

This study used a completely randomized design with seven dilution treatments. These treatments consisted of T0 = palm fruit juice (control); T1 = palmyra fruit juice + 10% egg yolk + 5% moringa leaf extract; T2 = palmyra fruit juice + 10% egg yolk + 10% moringa leaf extract; T3 = palmyra fruit juice + 10% egg yolk + 15% moringa leaf extract; T4 = palmyra fruit juice + 20% egg yolk + 5% moringa leaf extract; T5 = palmyra fruit juice + 20% egg yolk + 10% moringa leaf extract; and T6 = palmyra fruit juice + 20% egg yolk + 15% moringa leaf extract. Each treatment was repeated 4 times, yielding 28 experimental units.

Semen was diluted to a final concentration of 100×10^6 spermatozoa/mL. All diluted semen samples were stored in a cool box at 15–18°C. Semen quality was evaluated at 0, 24, 48, and 72 hours of storage. This study was designed as a controlled laboratory experimental study. Semen was obtained from a single superior Landrace boar to minimize genetic variability; however, this biological limitation may limit broader population generalization.

Research Procedures

Palmyra fruit juice was extracted under sterile conditions, used as the base diluent, then punctured; the juice was sucked out with a 20 mL syringe and collected in a sterile glass beaker. Egg yolks were prepared from fresh chicken eggs, which were first cleaned with 70% alcohol, dried, and cracked, and the whites were separated by weighing them on the shell.

The yolks were then rolled on sterile filter paper to remove any remaining white, punctured with a sterile needle, and collected in a glass beaker. Moringa leaf

extract was prepared at the Technical Implementation Unit of the Genetic Resources and Molecular Biology Laboratory of Udayana University using mature moringa leaves dried at room temperature for 3–5 days, then ground into a powder and macerated with 70% ethanol for 2 days. The maceration process was repeated until a clear macerate was obtained, which was then filtered and concentrated under vacuum in a rotary evaporator to form a paste.

The concentrated moringa leaf extract (0.5 g) was reconstituted in 50 mL of distilled water and homogenized using a magnetic stirrer at 400 rpm for 15 minutes. The solution was subsequently centrifuged at 1500 rpm for 2 × 30 minutes, and the resulting supernatant was used as the working extract solution in the diluent formulations [11]. The semen diluent was prepared by mixing palmyra fruit juice, egg yolk, and moringa leaf extract according to the composition of each treatment. Antibiotics (100,000 IU of penicillin and 100 mg of streptomycin) were added to prevent bacterial contamination. Semen from Landrace boars was collected using the gloved-hand technique, filtered through sterile gauze to remove the gel fraction, and maintained at 37°C prior to macroscopic and microscopic evaluation [2][12].

The semen and each diluent were equalized in a water bath at 37°C, then the semen was diluted to a final concentration of 100 × 10^6 spermatozoa/mL and stored in a cool box at 15–18°C. Semen quality evaluation was carried out at storage times of 0, 24, 48, and 72 hours, including intact plasma membrane (IPM), intact acrosomal membrane (IAM), progressive motility, viability, and spermatozoa abnormalities. Intact plasma membrane (IPM) was evaluated using the hypoosmotic swelling test (HOST) method with a hypoosmotic solution containing fructose and sodium citrate, incubated at 37°C for 45 minutes, and observed under a light microscope at 400× magnification on a minimum of 200 spermatozoa [13].

IPM examination was performed using a 0.9% NaCl solution containing formalin, and the preparations were observed under a microscope at 400× magnification, with the proportion of spermatozoa with intact acrosome caps calculated [4].

Progressive motility was evaluated by dropping semen on a warm glass object (37°C), observed at 400× magnification, and assessed in five fields of view [5][14].

Examination of spermatozoa viability and abnormalities was performed using eosin-nigrosin staining by observing 200 spermatozoa cells at 450× magnification, where live spermatozoa did not absorb color while dead spermatozoa were red, and abnormalities were determined based on morphological abnormalities in the head, neck, or tail of the spermatozoa [15-18].

Research Location and Timeline

This research was conducted at the Veterinary Reproductive Technology Laboratory, Faculty of Veterinary Medicine, Udayana University. Pig semen samples were collected at the Baturiti Artificial Insemination Center, Tabanan Regency, Bali. The study ran from February to November 2024.

Data Analysis

The obtained IPM, IAM, motility, vitality, and abnormality data were analyzed using Analysis of Variance (ANOVA) in SPSS version 25 for Windows. If there was a significant difference ($p<0.05$) in the treatment, the Duncan test was used to continue.

III. RESULTS AND DISCUSSION

Fresh Semen Quality from Landrace Pigs

This study used fresh semen from a single, healthy, 2.5-year-old superior Landrace pig. Semen collection was performed using a massage method with the aid of a dummy sow, and only the second fraction of ejaculate was used as the research sample. Macroscopic and microscopic evaluations of the fresh semen indicated it was of good quality and suitable for dilution (Table 1).

Macroscopically, the semen had an average volume of 185 mL, a creamy white color, medium consistency, a pH of 7.0, and a characteristic odor. Microscopic evaluation revealed mass motility (++), a spermatozoa concentration of 805 × 10^6 cells/mL, 80% progressive motility, 92% viability, and 5% sperm abnormalities. These results align with previous reports on fresh semen from Landrace pigs using only the second fraction of ejaculate, which showed relatively high and stable semen quality [8, 19]. Differences in semen volume and concentration reported in other studies are thought to be related to differences in ejaculate fractions used during semen collection [19-21]. The use of semen from a single boar reduces biological variability but limits extrapolation of results to broader boar populations, and this should be considered when interpreting the findings.

Intact Plasma Membrane (IPM)

Diluent formulation significantly affected plasma membrane integrity ($p < 0.05$). Treatments containing higher concentrations of egg yolk and moringa leaf extract demonstrated superior preservation effects, as reflected by higher IPM percentages compared with lower-concentration formulations and the control. The

combination of membrane-stabilizing phospholipids from egg yolk and antioxidant compounds from moringa leaf extract likely contributed to enhanced protection against cold-induced membrane damage.

Storage duration also significantly influenced IPM values, progressively declining from 88.38% at 0 hours to 70.02% at 24 hours, 51.86% at 48 hours, and 45.52% at 72 hours. The progressive reduction in membrane integrity during storage is consistent with increased oxidative stress and lipid peroxidation under hypothermic conditions, leading to structural destabilization of the sperm plasma membrane [10, 22].

The superiority of the T6 and T3 treatments is thought to stem from the optimal combination of egg yolk as a membrane protector and moringa leaf extract as an antioxidant source. Egg yolk contains phospholipids and cholesterol, which play a role in maintaining sperm membrane stability against cold shock [4, 5], while the antioxidant compounds in moringa leaf extract can suppress the formation of free radicals that damage membrane lipids [6, 7].

Intact Acrosome Membrane (IAM)

The diluent treatment had a significant effect ($p < 0.05$) on the percentage of intact acrosome membrane. The T6 and T3 treatments showed the highest IAM values, at 70.38% and 69.33%, respectively, and were not significantly different from each other. The pattern of IAM decline was consistent with the IPM results, with the control treatment (T0) showing the lowest value. Acrosomal membrane integrity is essential for the acrosome reaction during fertilization; oxidative damage may cause premature acrosomal enzyme leakage, thereby reducing fertilizing potential.

Storage duration also resulted in a significant decrease in IAM. The IAM value decreased from 88.79% at 0 hours to 70.29% at 24 hours, 51.93% at 48 hours, and 45.79% at 72 hours of storage. Acrosomal membrane damage is associated with lipid peroxidation and leakage of acrosomal enzymes, which play a crucial role in the fertilization process [23-25].

Progressive Sperm Motility

Progressive sperm motility was significantly affected by diluent type and storage duration ($p < 0.05$). The T6 treatment produced the highest progressive motility value (58.33%), followed by T3 and T5. Conversely, the control treatment showed the most drastic decrease in motility.

The progressive decrease in motility with increasing storage time indicates a close relationship between membrane damage, decreased energy supply, and an increase in the number of dead sperm [26]. Based on national standards, liquid semen with progressive motility ≥40% is still suitable for artificial insemination. Therefore, in this study, semen treated with T6 still met the criteria for up to 48 hours of storage.

Spermatozoa Viability and Abnormalities

The diluent treatment significantly affected ($p < 0.05$) spermatozoa viability and abnormalities. The T6 treatment resulted in the highest viability (70.08%) and the lowest abnormality rate (6.17%). Abnormality values for all treatments were still below the maximum allowable limit for boar semen.

The low abnormality rate in Moringa leaf extract-treated sperm suggests that antioxidants play a role in maintaining the structural integrity of spermatozoa during storage. Low spermatozoa abnormalities are crucial because abnormal spermatozoa are less likely to optimally fertilize an ovum [27-28].

Table 1.
Macroscopic And Microscopic Evaluation of Fresh Landrace Boar

Kualitas Semen Babi Landrace		
Macroscopic Evaluation	Semen consistency	Moderate
	Semen color	White–cream
	Semen volume (mL)	185
	pH	7.0
	Odor	Characteristic
Microscopic Evaluation	Mass movement	++
	Sperm concentration ($\times 10^6$/mL)	805
	Progressive motility (%)	80 (P)
	Live spermatozoa (%)	92
	Sperm abnormality (%)	5

Notes: ++ = good wave-like mass movement; P = rapid and progressive individual sperm movement.

Compared with 10% formulations, treatments containing 20% egg yolk consistently outperformed them, indicating that membrane stabilization plays a dominant role during hypothermic storage. However, without adequate antioxidant supplementation (as observed in lower moringa concentrations), membrane protection alone was insufficient to fully prevent oxidative decline. This suggests that optimal preservation requires both structural stabilization and oxidative stress mitigation working synergistically.

IV. CONCLUSIONS

In conclusion, the palmyra fruit water–egg yolk diluent supplemented with 15% moringa leaf extract significantly improved and preserved boar semen quality during liquid storage at 15–18°C. This formulation demonstrated superior membrane integrity, motility, and viability with minimal abnormalities compared to other treatments. Semen diluted with this formulation maintained acceptable quality for artificial insemination for up to 48 hours of storage.

Further research is recommended, including field trials of the use of palm fruit juice + 20% egg yolk + 15% moringa leaf extract as a diluent in pig artificial insemination programs, specifically to evaluate pregnancy rates and litter size. Furthermore, further research with varying concentrations of moringa leaf extract and longer storage periods can be conducted to optimize the formulation of natural-based pig semen diluents.

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CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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