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Effects of Various Tris Citrate Fructose-based Extenders on Plasma Membrane Integrity, Sperm DNA Fragmentation, and Artificial Insemination Success in Pigs

(*Pengaruh Berbagai Pengencer Berbasis Tris-Sitrat-Fruktosa terhadap Keutuhan Membran Plasma, Fragmentasi DNA Sperma, dan Keberhasilan Inseminasi Buatan pada Babi*)

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ABSTRAK

Keberhasilan reproduksi merupakan faktor kunci dalam meningkatkan efisiensi dan produktivitas usaha peternakan babi. Salah satu strategi yang digunakan untuk mendukung keberhasilan tersebut adalah inseminasi buatan (IB), yang sangat bergantung pada kualitas semen selama penyimpanan. Penelitian ini bertujuan untuk mengevaluasi integritas membran plasma (IMP), fragmentasi DNA spermatozoa, tingkat keberhasilan IB, dan *litter size* menggunakan semen babi yang diencerkan dengan berbagai jenis pengencer berbasis tris sitrat fruktosa (TCF), selanjutnya disimpan pada suhu 16 °C hingga 72 jam. Penelitian dilakukan dalam dua tahap. Tahap pertama menilai kualitas semen, yaitu PMI dan fragmentasi DNA, menggunakan rancangan acak lengkap dengan lima ulangan. Tiga jenis pengencer yang diuji adalah TCF + ekstrak mesokarp + kuning telur (TCF+EM+KT), TCF + ekstrak mesokarp (TCF+EM) dan TCF, masing-masing pada penyimpanan 24, 48, dan 72 jam. Tahap kedua mengevaluasi keberhasilan IB pada 36 ekor induk babi hasil persilangan *Duroc-Landrace* berumur 1–3 tahun, menggunakan semen yang disimpan selama 72 jam. Parameter yang diamati meliputi *conception rate* (CR), *service per conception* (S/C), *non-return rate* (NRR), dan ukuran *litter*. Hasil penelitian menunjukkan bahwa pengencer TCF+EM+KT memberikan hasil terbaik, dengan nilai IMP tertinggi (97,4±0,43%), fragmentasi DNA terendah (0,40±0,55%), CR tertinggi (83%), S/C terendah (1,17), serta rata-rata *litter size* terbesar (7,42), secara signifikan berbeda ($P<0,05$) dibandingkan pengencer lainnya. Kesimpulan penelitian ini menunjukkan pengencer TCF+EM+KT paling efektif dalam mempertahankan kualitas semen dan meningkatkan performa reproduksi pada IB babi.

Kata Kunci : fragmentasi DNA; inseminasi buatan; *litter size*; membran plasma utuh; pengencer tris sitrat fruktosa; tingkat kebuntingan

INTRODUCTION

Artificial insemination (AI) in sows is highly dependent on semen quality, a critical factor influencing conception rates and litter size. Maintaining semen quality during storage requires an effective extender to preserve spermatozoa viability. Boar sperm is particularly vulnerable to oxidative stress at low temperatures, which can damage the acrosome, DNA, and plasma membrane leading to reduced fertilization capacity and reproductive performance (Pintus & Ros-Santaella, 2021). Oxidative stress caused by reactive oxygen species (ROS) is a major contributor to acrosomal and DNA damage (Pezo *et al.*, 2021). The acrosome contains enzymes for oocyte penetration, while DNA damage compromises embryo genetic integrity. The plasma membrane is equally vital, supporting motility and survival in the female reproductive tract.

The TCF-based extenders are known to stabilize pH and supply energy to spermatozoa. The addition of natural antioxidants, such as palm extract rich in polyphenols and flavonoids, may enhance antioxidant defence and protect sperm structures (Parera & Lenda, 2023). Egg yolk, with its high phospholipid and lipid content, also helps stabilize the plasma membrane and sustain sperm viability (Bustani & Baiee, 2021). This study aimed to evaluate plasma membrane integrity, DNA fragmentation, AI success rate, and litter size in pigs using different semen extenders stored at 16 °C for 72 hours.

MATERIALS AND METHODS

This study consisted of two stages: (1) evaluation of semen quality, including PIM and DNA fragmentation, and (2) evaluation of AI success based on conception rate (CR), service per conception (S/C), non-return rate (NRR), and litter size. Semen was collected from three boars of Duroc and Duroc-Landrace crossbreeds aged 1.5–2 years, raised by local farmers. Collections were carried out twice weekly, and initial evaluations were conducted at the Laboratory of Anatomy and Pathology, State Agricultural Polytechnic of Kupang. Only fresh semen with progressive motility ≥70% and morphological abnormalities ≤20% (BSN, 2023) was used.

The study used a completely randomized factorial design with five replications. The treatments consisted of two factors: storage duration (24, 48, and 72 hours) and type of extenders: (1) Tris Citrate

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Fructose (Merck® Cat. No. 106448) supplemented with 0.01% palm mesocarp extract (EM) and 5% egg yolk (TCF+EM+EY); (2) TCF with 0.01% EM (TCF+EM); and (3) TCF without any additional substances (TCF). The extender was composed of tris(hydroxymethyl)aminomethane (Merck® Cat.No. 108387), citric acid monohydrate (Merck® Cat.No.1002440500), fructose (Himedia® Cat.No. GRM1355-500G), EM, egg yolk, distilled water, and antibiotics. Semen was diluted according to the test groups and stored at 16 °C for up to 72 hours. Daily evaluations were conducted to assess motility, viability, PMI, and DNA fragmentation.

The integrity of the sperm plasma membrane was assessed using Hypo-Osmotic Swelling Test (HOST) as described by Check *et al.*, (2023). The HOST solution was prepared using sodium citrate and fructose dissolved in distilled water. A total of 0.1 mL of semen was mixed with 0.9 mL of the HOST solution and incubated for 15 minutes at room temperature. After incubation, a drop of the mixture was placed on a microscope slide, smeared, and air-dried. The smear was then observed under a digital microscope (Hirox®KH-8700) at 400× magnification. The percentage of spermatozoa with intact plasma membranes was determined by counting the number of cells exhibiting curled tails relative to the total number of sperm cells observed.

DNA fragmentation was evaluated following the method of Priyanto *et al.* (2015). Semen samples were smeared on slides, air-dried, and fixed in a 1:1 solution of 96% ethanol and acetone for 30 minutes at 4 °C. After fixation, the slides were hydrolysed in 0.1 N HCl for five minutes at the same temperature, then rinsed three times with distilled water. The preparations were stained with 0.05% toluidine blue (TB) (Himedia® CAS No. 92-31-9) for 10 minutes, rinsed again with distilled water, dehydrated twice using t-butanol, and cleared twice with xylene. Observations were made using a digital microscope at 400× magnification. Spermatozoa with good chromatin integrity appeared light blue, whereas those with fragmented DNA appeared dark blue.

The second phase involved AI technique of 36 Duroc-Landrace crossbred, aged 1–3 years, each with at least one prior parturition. AI success was evaluated using four parameters: S/C, CR, NRR and litter size. S/C was calculated as the total number of AI doses divided by the number of pregnant sows. CR was determined as the percentage of sows that became pregnant after one insemination cycle. NRR indicated the proportion of sows that did not

return to estrus within 21 days post-AI, suggesting early pregnancy. Litter size referred to the total number of piglets born per farrowing.

Parametric data of S/C, CR, NRR and litter size were analyzed by ANOVA test and differences among treatments were followed by Duncan's Multiple Range Test (Steel and Torrie, 1995).

RESULTS AND DISCUSSION

Plasma Membrane Integrity (PMI) of Boar Spermatozoa

The integrity of the plasma membrane (PMI) in boar spermatozoa is a critical indicator of semen quality and its fertilization potential. The plasma membrane protects from mechanical and oxidative damage, thus playing a vital role in maintaining sperm viability and motility (Gautier & Aurich, 2022), and can disrupt the physiological functions of spermatozoa during artificial insemination (AI), potentially lowering conception rates (Wysokińska & Szablicka, 2021). Data on the percentage of PMI in boar spermatozoa stored at 16 °C are presented in Table 1.

Table 1. Percentage of intact plasma membrane of boar sperm

Types of Diluents	Storage Time at Temperature 16 °C		
	L24	L 48	L 72
TCF+EM+KT	97.4 ± 0.43^{aA}	94.4 ± 0.43^{aB}	90.8 ± 0.43^{aC}
TCF+EM	96.6 ± 0.43^{bA}	93.2 ± 0.43^{bB}	89.8 ± 0.43^{bC}
TCF	94.2 ± 0.43^{cA}	88 ± 0.43^{cB}	85.2 ± 0.43^{cC}

Different superscripts (a, b, c) within the same row indicate significant differences ($P < 0.05$); different superscripts (A, B, C) within the same column indicate significant differences ($P < 0.05$)

Based on Table 1, the TCF+EM+KT group consistently maintained the highest percentage of PMI throughout 72 hours of storage. Although a decline was observed over time, the values remained significantly higher ($P<0.05$) compared to the other groups. This group showed the best performance in preserving the PMI during storage at 16 °C, despite the time-related decline. The decrease in PMI is presumed to result from oxidative stress during low-temperature storage, which can damage the plasma membrane. Palm mesocarp extract, rich in polyphenols and flavonoids, along with egg yolk, which contains abundant phospholipids, are known for their antioxidant properties. These components can protect spermatozoa from oxidative damage. This finding aligns with previous research indicating that antioxidants are effective in slowing down spermatozoa degradation,

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maintaining PIM, and preserving motility during long-term storage (Kowalczyk, 2022).

DNA Fragmentation in Boar Spermatozoa

DNA fragmentation is a key parameter for evaluating the genetic quality of spermatozoa, as it can significantly affect fertilization and subsequent embryo development (Agarwal *et al.*, 2020). The percentage of DNA fragmentation in boar spermatozoa during storage in this study is presented in Table 2.

The results in Table 2 show that DNA fragmentation in spermatozoa increased with longer storage duration. The TCF+EM+KT group consistently exhibited the lowest DNA fragmentation levels up to 72 hours, with a statistically significant difference ($P<0.05$) compared to the other two groups. In this group, DNA fragmentation was recorded at 0.40±0.548% during 24–48 hours of storage and increased to 1.20±0.837% at 72 hours. The TCF+EM group showed a fragmentation level of 0.60±0.548% at 24–48 hours, rising to 2.00±0.707% at 72 hours. Meanwhile, the TCF group showed the highest fragmentation, starting at 0.80±0.548% (24 hours), increasing to 1.60±1.14% (48 hours), and reaching 2.40±0.704% at 72 hours.

Table 2. Percentage of boar sperm DNA fragmentation

Types of Diluents	Storage Time at Temperature 16 °C		
	L24	L 48	L 72
Damaged DNA			
TCF+EM+KT	0.40±0.548^{a}	0.40±0.548^{a}	1.20±0.837^{a}
TCF+EM	0.60±0.548^{a}	0.60±0.548^{a}	2.00±0.707^{a}
TCF	0.80±0.548^{b}	1.60±1.14^{b}	2.40±0.548^{a}
Intact DNA			
TCF+EM+KT	99.6±0.306^{aA}	99.6±0.306^{aA}	98.8±0.306^{aB}
TCF+EM	99.4±0.306^{aA}	99.4±0.306^{aA}	98 ±0.306^{aB}
TCF	99.2±0.306^{bA}	98.4±0.488^{bA}	97.6±0.306^{bB}

Different superscripts (a, b, c) within the same row indicate significant differences ($P < 0.05$); different superscripts (A, B, C) within the same column indicate significant differences ($P < 0.05$)

The effectiveness of the TCF+EM+KT extender in preserving DNA integrity is likely due to the synergistic action of egg yolk and EM, both rich in phospholipids and antioxidants, such as β-carotene. These compounds play a role in reducing oxidative stress and protecting sperm DNA (Kowalczyk, 2022). Protection against ROS can maintain membrane stability and cell viability during storage (Kumaresan *et al.*, 2020). In contrast, the absence of egg yolk in the TCF+EM extender reduced its

protective capacity, although the palm extract still provided partial antioxidant effects. Nevertheless, according to Parera *et al.* (2024), semen stored with the TCF extender alone remains within the acceptable safety limits for artificial insemination. However, the increase in DNA fragmentation across all groups indicates that prolonged storage may accelerate oxidative stress accumulation, ultimately compromising the genetic integrity of spermatozoa (Silvestre *et al.*, 2021).

Artificial Insemination Success Rate

The success of artificial insemination (AI) in pigs can be evaluated using several reproductive performance indicators, including S/C, CR, NRR, and litter size. The results related to these four parameters are presented in Table 3.

The TCF+EM+KT extender group have the lowest S/C value at 1.17, compared to TCF+EM (1.36) and TCF (1.78). An S/C value close to one indicates high reproductive efficiency, as only a single insemination is required to achieve pregnancy. These results suggest that the combination of TCF, egg yolk, and EM effectively enhances fertilization efficiency by preserving semen during storage. In contrast, the TCF group exhibited the highest S/C value, indicating the need for more insemination doses. This suggests that the TCF extender without additional supplements is less effective in maintaining semen quality during storage. These findings differ from Kaka (2020), who reported an S/C value of 1.03 in Landrace pigs using fresh, unstored semen. According to Table 3, the TCF+EM+KT group achieved the highest CR and NRR values (83%), followed by TCF+EM (75%) and TCF (67%), indicating that TCF+EM+KT was the most effective in improving pregnancy outcomes. Kim *et al.* (2021) noted that, in general, a good conception rate in pigs ranges from 80% to 90%.

Table 3. S/C, CR, NRR, and litter size using different semen diluents

Group Thinner	Number of sows	First AI Pregnant	Total AI Doses	Total Pregnant	S/C	CR1	NRR1	Average Litter size
TCF+EM+KT	12	10	14	12	1.17	83%	83%	7.42[a]
TCF+EM	12	9	15	11	1.36	75%	75%	6.0[b]
TCF	12	8	16	9	1.78	67%	67%	4.75[c]

[a b c] Different superscripts within the same row indicate significant differences ($P < 0.05$)

The CR and NRR is strongly influenced by semen quality, which depends on the composition of the extender. The TCF extender,

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enriched with egg yolk and EM has been shown to effectively preserve spermatozoa quality during low-temperature storage for >72 hours. Fructose serves as an energy source, tris as a pH buffer, and egg yolk, rich in phospholipids and cholesterol, helps protect the cell membrane against cold shock damage (Namula *et al.*, 2019; Wysokińska & Szablicka, 2021). Palm mesocarp extract contains antioxidants such as β-carotene, which can inhibit the formation of ROS, thereby preventing lipid peroxidation and maintaining plasma membrane integrity (Parera & Lenda, 2023).

Litter size is a key indicator of reproductive performance in pigs. According to Table 3, a significant difference ($P<0.05$) was observed among treatments. The TCF+EM+KT group showed the highest average litter size at 7.42 piglets per sow (totalling 89 piglets), compared to TCF+EM (6.00) and TCF (4.75). Although slightly lower than the findings of Lotu *et al.* (2017), reported averages of 8.8 piglets in Duroc-cross, the use of TCF+EM+KT still demonstrated a positive contribution to fertilization success and embryo development. The increased litter size is likely the result of the synergistic effects of fructose, tris, egg yolk, and palm mesocarp extract in preserving spermatozoa viability and integrity during storage. In addition to semen quality, litter size is also influenced by factors such as breed, timing of insemination, parity, nutrition, environment, health status, and reproductive genetics (Buthelezi *et al.*, 2024).

CONCLUSION

The TCF+EM+EY extender proved to be the most effective in enhancing reproductive efficiency in pigs. This formulation was able to preserve spermatozoa PIM, prevent DNA fragmentation, and result in higher pregnancy rates and litter sizes. The combination of egg yolk and antioxidant-rich palm mesocarp extract provided optimal protection for sperm quality, supported the fertilization process, and improved overall reproductive performance.

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