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Application of Plant-Based Dyes for Acrosomal Cap Visualization in Cauda Epididymal Spermatozoa of Bali Cattle

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ABSTRACT

The cauda epididymis contains mature spermatozoa capable of fertilization, with acrosomal integrity being a crucial factor in their function. Damage to the acrosome impairs the viability and fertilizing ability of spermatozoa. Conventional staining methods, such as Giemsa or fluorescence microscopy, are effective but less practical in low-resource settings. The present study aimed to evaluate damage to the acrosomal cap of cauda epididymal spermatozoa in Bali cattle and to assess the integrity of the acrosomal cap using Giemsa and natural dyes such as turmeric, beetroot, and areca nut extract. The present study utilized cauda epididymal spermatozoa from Bali cattle aged 3-4 years and weighing 316-412 kg. The study employed a completely randomized design with four treatments, each replicated 20 times. The treatments were Giemsa stain 100% (Control, P0), 10% Giemsa + 90% turmeric extract (P1), 10% Giemsa + 90% beetroot extract (P2), and 10% Giemsa + 90% areca nut extract (P3). Using Giemsa and natural dyes resulted in different colors of spermatozoa acrosomes. The P0 preparations exhibited a dark purple coloration, P1 appeared yellowish, corresponding to the turmeric pigment, and P3 appeared bluish, while P2 demonstrated no detectable staining on the slides. The acrosomal caps of spermatozoa in P0 were dark purple, those in P1 were yellowish, and those in P2 and P3 were unstained (Transparent or faint). The average acrosomal cap integrity was the highest in P0, followed in order by P1, P2, and P3. Based on the current findings, it can be concluded that P1, P2, and P3 signified acrosomal cap integrity in cauda epididymal spermatozoa of Bali cattle. However, P1 produced the best results, showing a distinguishable color and the highest acrosomal cap integrity score (86.9 ± 2.0%). Beetroot and areca nut extracts produced weaker staining and lower acrosomal integrity values. Therefore, turmeric extract may serve as a practical, cost-effective, and environmentally friendly alternative for acrosome evaluation in veterinary reproductive laboratories, particularly in resource-limited settings.

Keywords: Giemsa, Intact acrosomal cap, Natural dye, Spermatozoa

INTRODUCTION

Slaughterhouse operations involving cattle not only produce meat but also generate several by-products, including reproductive organs, which are often discarded as waste. The waste products generated in slaughterhouses include the testis and epididymis, which represent reproductive tissues with untapped potential, particularly as sources of viable spermatozoa that remain functional for 24 to 48 hours when stored appropriately in a refrigerator at 5°C (Abu et al., 2016). The epididymis is anatomically divided into three regions, including caput, corpus, and cauda, with the cauda epididymis serving as the primary reservoir for mature spermatozoa before ejaculation (James et al., 2020). The success of fertilization within the female reproductive tract is heavily dependent on the quality of the spermatozoa, particularly the structural and functional integrity of the acrosomal cap (He et al, 2022). The acrosome, which overlays the spermatozoa nucleus, contains hydrolytic enzymes such as hyaluronidase and acrosin, which are essential for penetrating the zona pellucida during fertilization (He et al., 2022). Preservation of acrosomal integrity is vital; damage to the acrosomal spermatozoa structure impairs spermatozoa function and may compromise fertilization, ultimately affecting conception rates (Sun et al., 2020).

Acrosomal damage may result from alterations in the lipid composition of the spermatozoa membrane, disrupting its stability and increasing susceptibility to structural degeneration (Tethool et al., 2022). Moreover, defects in acrosome formation may arise from abnormalities in spermatogenesis, particularly due to the failure of the Golgi apparatus in spermatids to produce a functional acrosome. For successful fertilization, spermatozoa should possess an intact acrosome capable of undergoing a timely acrosome reaction, which facilitates the release of enzymes such as acrosin, hyaluronidase, and corona-penetrating enzymes, thereby enabling spermatozoa penetration through the zona pellucida (Khan et al., 2024). Assessment of acrosomal integrity is commonly performed using the chlortetracycline fluorescence

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staining technique (Baňas et al., 2024). While effective, the evaluation of acrosomal integrity using chlortetracycline fluorescence requires advanced laboratory equipment, such as a fluorescence microscope, and is therefore less suitable for routine or field-based applications (Kumar et al., 2019). Giemsa staining is a widely used cytological technique for visualizing cellular components, including the acrosomal structure. While Giemsa is considered a reliable agent for assessing acrosomal integrity, its application may be constrained by high costs and limited availability, particularly in remote or resource-limited laboratory settings (Kútvölgyi et al., 2006). The limitations associated with Giemsa staining have encouraged the search for alternative staining solutions utilizing natural pigments that are more accessible, cost-effective, and environmentally sustainable (Chowdhury et al., 2014; Alegbe and Uthman, 2024).

Natural dyes such as those extracted from turmeric (*Curcuma longa*), areca nut (*Areca catechu*), and beetroot (*Beta vulgaris*) have demonstrated potential as biological stains (Nirmala and Puspitasari, 2017; Obeta et al., 2022). Turmeric contains curcuminoids, primarily curcumin, desmethoxycurcumin, and bisdemethoxycurcumin, which provide an intense yellow pigment and have been used to stain plant tissues effectively (Amalraj et al., 2017; Manasa et al., 2023). Areca nut is rich in flavonoid pigments that impart a deep orange color, and its extract has yielded clear and contrasting tissue staining results (Sharan et al., 2012). Beetroot contains betalain pigments, specifically betacyanin and betaxanthins, which offer vibrant red to purple coloration and possess potent antioxidant properties, including high vitamin C content (Liliana and Oana-Viorela, 2020). Given their biochemical properties and natural pigment content, these plant-based extracts have the potential to serve as alternative staining agents for acrosomal evaluation. The application of natural dye extracts could facilitate the development of simpler, safer, and more sustainable staining protocols that are better suited for use in veterinary laboratories, particularly in resource-limited settings (Alegbe and Uthman, 2024). The present study aimed to evaluate the effectiveness of turmeric, areca nut, and beetroot as natural dyes in identifying the acrosomal cap and assessing the acrosomal integrity of cauda epididymal spermatozoa in Bali cattle, to establish a more practical and environmentally friendly alternative to conventional staining methods such as Giemsa.

MATERIALS AND METHODS

Ethical approval

All procedures carried out in the present study complied with the operational protocols of the slaughterhouse located in Manokwari Regency, West Papua, Indonesia. The study was conducted with official research approval under permit number 061/UN42.3/PP/2023. Cauda epididymal tissues were obtained following the completion of the slaughter process.

Animals and materials

The present study utilized cauda epididymal spermatozoa from Bali cattle aged 3-4 years and weighing 316-412 kg. Cauda epididymal samples were obtained from 20 Balinese cattle slaughtered at the slaughterhouse in Manokwari Regency, West Papua, Indonesia. The cauda epididymis was placed in a thermos containing warm water (30-40 °C) and immediately transported to the laboratory for further observation. The materials used included Giemsa stain (Merck Millipore, Germany), methanol 70% (Merck Millipore, Germany), phosphate buffer (Bioenno Tech, USA), ethanol 90% (Merck Millipore, Germany), eosin-nigrosin (Merck Millipore, Germany), distilled water, and warm water. Natural staining agents were derived from turmeric (*Curcuma xanthorrhiza* Roxb.), areca nut (*Areca catechu* L.), and beetroot (*Beta vulgaris* L.; Figure 1). Each plant material, weighing ± 500 grams, was procured from local sources in Manokwari, West Papua, Indonesia.

Figure 1. The used plants as natural dyes for acrosomal cap visualization in cauda epididymal spermatozoa of Bali cattle. **A**: Turmeric (*Curcuma xanthorrhiza* Roxb), **B**: Areca nut (*Areca catechu* L), and **C**: Beetroot (*Beta vulgaris* L.).

Experimental design

An experimental study was conducted using a completely randomized design with 20 Bali cattle aged 3-4 years. The study employed four treatments, each replicated 20 times, including 100% Giemsa stain solution (Control, P0), 10% Giemsa stain solution + 90% turmeric extract (P1), Giemsa stain solution 10% + beetroot extract 90% (P2), and Giemsa stain solution 10% + areca nut extract 90% (P3). The percentage of plant extract used was based on the studies of Hartika et al. (2021), Shankar et al. (2022), and Mulia et al. (2022), which demonstrated that the optimal staining for each spermatozoa preparation using plant-based dyes was achieved with a composition of 90% natural extract combined with 10% Giemsa solution. This percentage was applied to facilitate the preparation of the staining solution, with a total volume of 200 mL composed of 10% Giemsa staining solution and 90% of each respective natural dye.

Preparation of staining solutions

Giemsa solution

The Giemsa staining solution was prepared by slowly adding 3 mL of Giemsa to a standard phosphate buffer solution, followed by the addition of 35 mL of distilled water. The mixture was stirred until homogenized (Tethool et al., 2022).

Natural dye extracts

The natural dye solutions were prepared based on preliminary trials conducted by the study of Tethool et al. (2022) using the following steps. Initially, fresh turmeric and beetroot (each 500 grams) were peeled, washed, and cut into small pieces. Then, each was ground using a chopper, and the juice was extracted. A total of 20 mL of each extract was mixed with 10 mL of methanol, 10 mL of phosphate buffer, and 35 mL of distilled water. The areca nuts were crushed, added to 100 mL of distilled water, and heated. A 30 mL aliquot of areca nut extract was then combined with 10 mL phosphate buffer and 30 mL distilled water. The extraction process of each natural material resulted in a final volume of ± 40 mL.

Evaluation of cauda epididymal spermatozoa

Spermatozoa motility

To evaluate motility (%), a 5 µL aliquot of spermatozoa from the cauda epididymis was dispensed onto a microscope slide, followed by the addition of one drop of sodium chloride solution (Widarta Bhakti, Indonesia). The preparation was covered with a coverslip and examined under a light microscope (Olympus CX 31, Japan) at 400x magnification (Gustina et al., 2022).

Spermatozoa concentration

A volume of 10 µL semen was mixed with 990 µL of sodium chloride solution in a microtube to achieve dilution. From the semen and sodium chloride mixture, 10 µL was drawn and placed into both chambers of a hemocytometer (Marienfeld, Germany). Spermatozoa concentration ($x10^7$/mL) was determined by averaging the counts from two separate grid areas (Santoso et al., 2021; Cipriani et al., 2023).

Spermatozoa viability

Assessment of spermatozoa viability (%) was carried out using eosin-nigrosin staining. A 5 µL semen sample was combined with 20 µL of eosin-nigrosin solution on a glass slide, mixed evenly, and then smeared into a thin layer for evaluation (Tethool et al., 2022). Spermatozoa viability assessment was performed using a microscope (Olympus CX 31, Japan) set at 400x magnification.

Spermatozoa abnormality

A thin smear was prepared using eosin-nigrosin-stained semen and examined under a microscope (Olympus CX 31, Japan) at 400x magnification. Spermatozoa abnormalities (%) were calculated as the percentage of abnormal spermatozoa from a total of 200 spermatozoa, including abnormalities of the head and tail (Suhardi et al., 2020).

Acrosomal integrity

Spermatozoa were collected from the dissected cauda epididymis, smeared onto glass slides, and air-dried for approximately five minutes. Slides were fixed in formalin 5% (Merck, Germany) for 30 minutes at 37°C, rinsed with distilled water, air-dried again, and then immersed in Giemsa stain for 4 hours at 37°C (Chowdhury et al., 2014; Prihantoko et al., 2020).

Statistical analysis

All data were analyzed using SPSS software version 25.0 (IBM). A one-way ANOVA was performed to assess differences among treatments, followed by Duncan's post hoc test. Statistical significance was set at a p-value less than 0.05 ($p < 0.05$).

RESULTS AND DISCUSSION

Color characteristics of spermatozoa stained by natural dyes

Samples that were stained using Giemsa and natural dyes exhibited distinct coloration (Figure 2). The control group (P0) stained with Giemsa appeared dark purple (Figure 2A), while preparations treated with turmeric extract (P1) were yellowish, consistent with the natural pigment content of turmeric (Figure 2B). Group P2, which used beetroot extract, demonstrated no detectable coloration (Figure 2C), while P3 preparation appeared bluish (Figure 2D). According to Nemzer et al. (2011), beetroot contains the red pigment betacyanin; however, in the present study, the combination of beetroot extract and Giemsa resulted in a diluted base color, namely red. Furthermore, the concentration of beetroot extract used was relatively low, approximately 31 mL, leading to a translucent appearance. The coloration observed in each preparation, such as turmeric extract stain (P1), beetroot extract stain (P2), and areca nut stain (P3), reflected the presence of active compounds in the dye materials. The yellowish tint in P1 is attributable to curcuminoids in turmeric (Ciuca and Racovita, 2023). These phenolic compounds, comprising curcumin, monodesmethoxycurcumin, and bisdemethoxycurcumin, are responsible for the yellow staining (Basha et al., 2024).

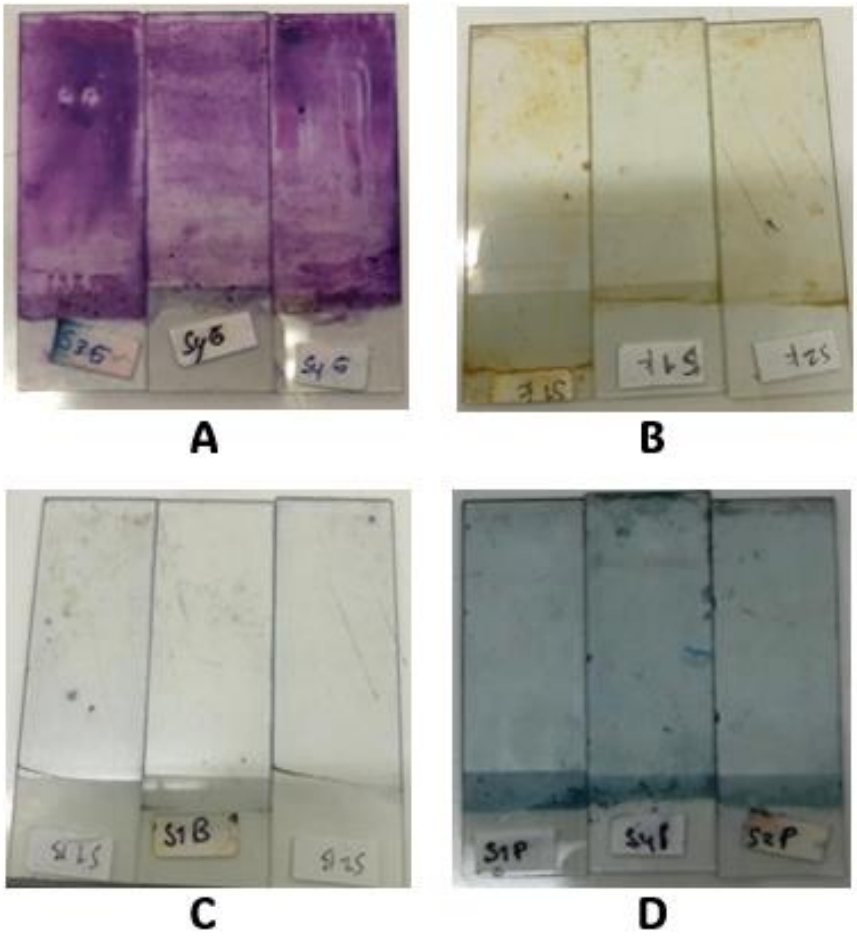

Figure 2. Staining of a 3-4-year-old Bali cattle sperm using natural dyes. **A**: Giemsa staining, **B**: Turmeric extract staining, **C**: Beetroot extract staining, and **D**: Areca nut extract staining.

According to Rautela et al. (2020), preparations stained with Giemsa for acrosomal cap evaluation showed a similar purple color as observed in the present study (Figure 2A). Preparation P3 exhibited a bluish hue, which contrasts with the findings of Ati et al. (2010), who reported that areca nut pigments (Flavonoids) produce an orange coloration. The observed difference is presumably due to the dominant staining effect of Giemsa, which overrides the natural pigment of areca nut. Additionally, preparations stained with beetroot extract demonstrated only a faint and transparent coloration. Acrosomal cap coloration varied among treatments in P0 and P1 (Figure 3A and 3B). Preparations stained with Giemsa (P0) produced a clearly defined purple acrosomal cap (Figure 3A), as Giemsa binds to membrane proteins of the sperm (Prihantoko et al., 2020). Spermatozoa in preparation P1 exhibited a yellowish acrosomal cap (Figure 3B), resulting from the turmeric extract. The acrosomal cap was distinctly visible when stained with turmeric dye in P1 treatment. In contrast, P2 and P3 produced pale or transparent caps (Figures 3C and 3D), indicating possible acrosomal damage. This

is supported by Tethool et al. (2022), who noted that damage to the spermatozoa acrosome results in faded or pale staining.

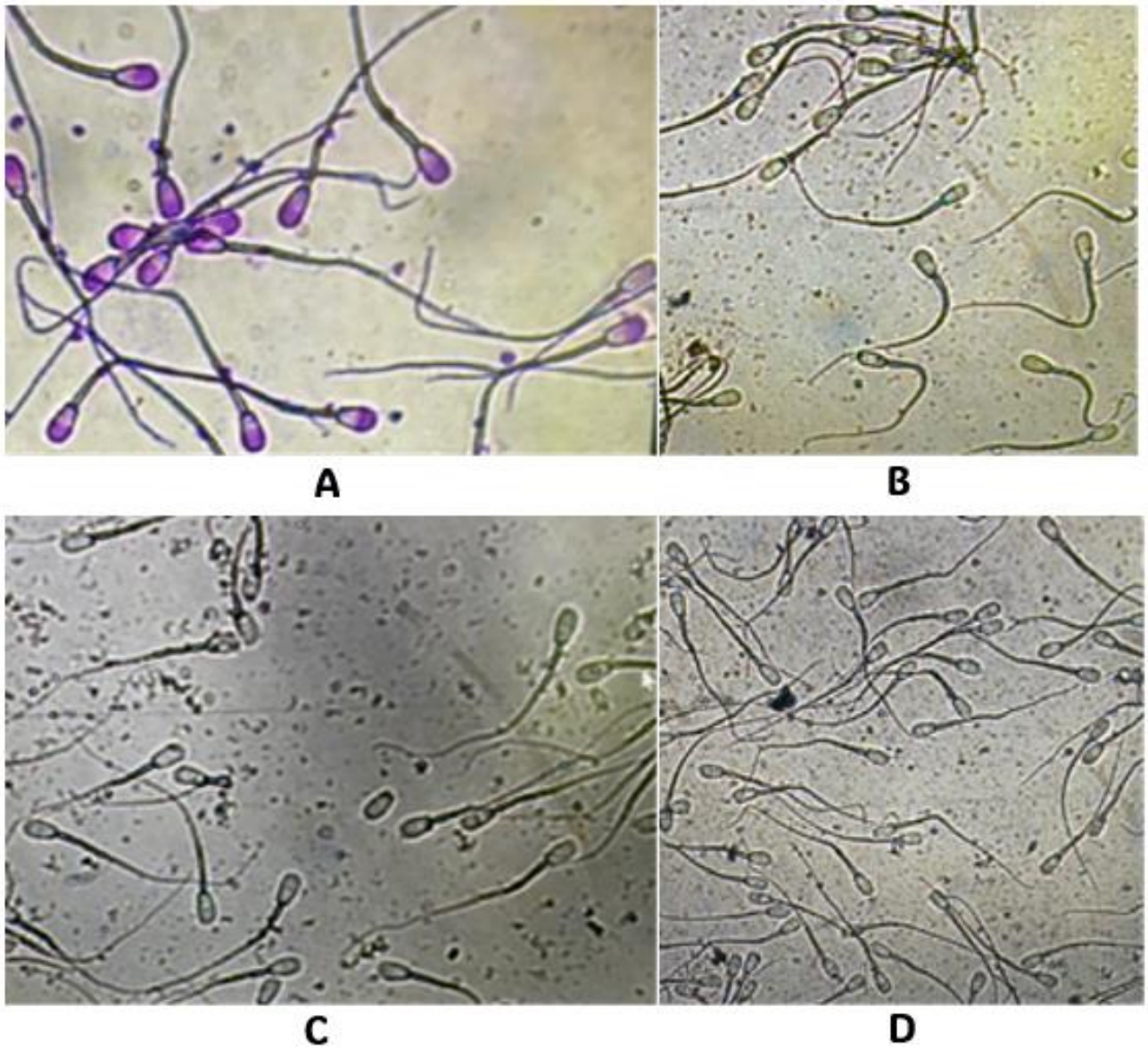

Figure 3. Acrosomal cap staining of a 3-4-year-old Bali cattle spermatozoa using natural dyes. **A**: Giemsa staining, **B**: Turmeric extract staining, **C**: Beetroot extract staining, **D**: Areca nut staining.

Integrity value of the spermatozoa acrosomal cap

An intact acrosomal cap plays a vital role in spermatozoa functionality, as it reflects their competence to recognize and adhere to the zona pellucida of the oocyte, trigger the acrosome reaction, and facilitate the enzymatic processes required for fertilization (Leung et al., 2023). Fertilizing capability is influenced by parameters such as motility, membrane integrity, and acrosome status (Bernecic et al., 2021). The acrosome plays a critical role in fertilization, as its interaction with the zona pellucida triggers the release of enzymes such as acrosin, hyaluronidase, and corona-penetrating enzymes, allowing spermatozoa to penetrate (Hirohashi and Yanagimachi, 2018).

The percentage of intact acrosomal caps detected with natural stains showed a significant difference between P1 and P3 ($p < 0.05$). Among all treatment groups, the Giemsa-stained group (P0) indicated the highest mean percentage ($87.4 \pm 2.7\%$), with a slightly lower value observed in P1 ($86.9 \pm 2.0\%$). In contrast, the mean percentages in P2 and P3 were comparatively lower, at $84.5 \pm 5.6\%$ and $84.4 \pm 4.3\%$, respectively (Figure 4). Giemsa is known to have a strong affinity for proteins, enabling it to stain the acrosome of spermatozoa effectively. The specific proteins, such as serine protease, the acrosin-binding protein (Sp32), and β-galactosidase (Nadgas et al., 2016), in the spermatozoa membrane bind to the dye, enabling clear visualization and differentiation. The acrosome, located at the anterior portion of the spermatozoa head, is essential as it contains key enzymes required for oocyte penetration during fertilization. Intact acrosomal membranes are crucial for maintaining the fertilization potential of spermatozoa (Fannessia et al., 2015).

Damage to the acrosome, particularly to its membrane, can compromise fertilization capability, reduce motility, and lead to cell death.

A cost-effective and straightforward method to evaluate acrosomal integrity involves Giemsa staining (Kumar et al., 2018). In spermatozoa with intact acrosomes, Giemsa produces a more intense (Dark purple) staining in the acrosomal region, indicating membrane integrity and preserved protein structure. Conversely, spermatozoa with damaged acrosomes exhibit fainter or unclear staining (Light purple), reflecting compromised membrane integrity (Prihantoko et al., 2020). In contrast, the staining ability of natural dyes is influenced by their varied chemical composition, such as carbohydrates, proteins, minerals, essential oils (Phellandrene, sabine, cineol, borneol, zingiberene, and sesquiterpenes; Athala, 2021; Hartika et al., 2021). In cases of acrosomal damage, staining efficiency may be compromised, leading to weak or inconsistent coloration. Additionally, morphological abnormalities, particularly in the spermatozoa head region, can indicate disruptions in the plasma membrane and acrosomal integrity (Esteves, 2007).

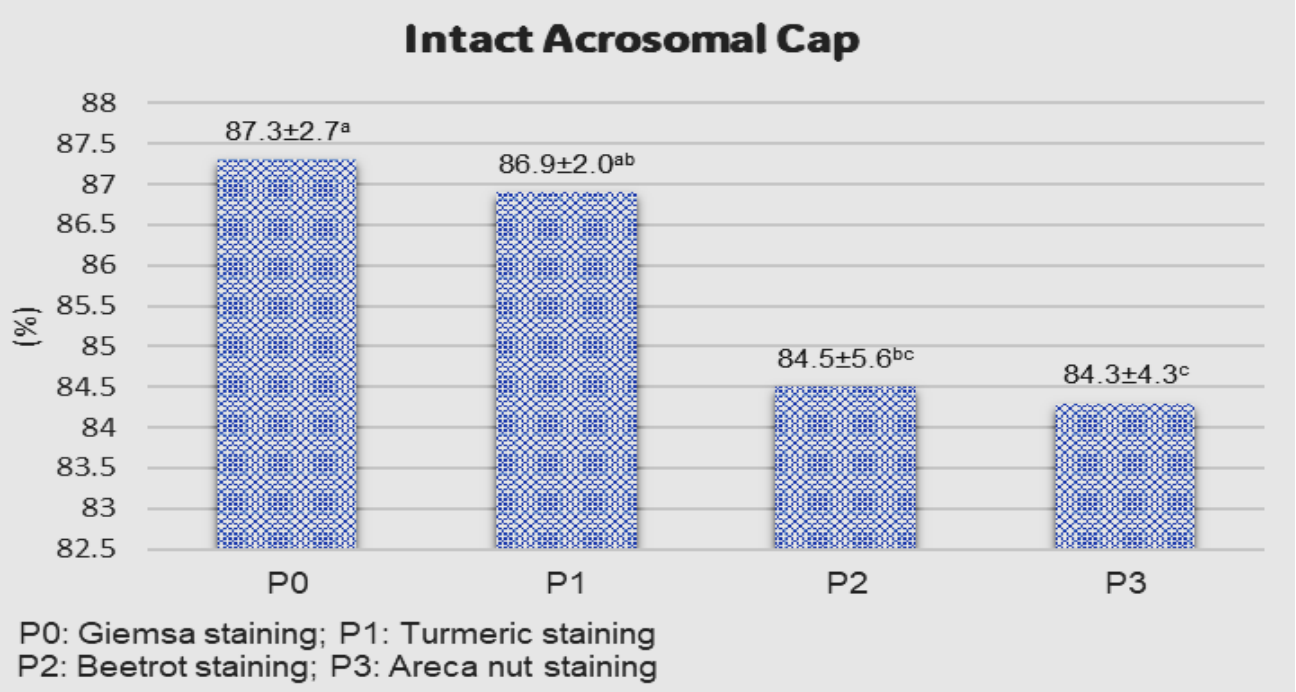

Figure 4. The percentage of intact acrosomal caps in Bali cattle (3-4 years old) across different treatments. [a,b,c] Different superscript letters indicate significant differences ($p < 0.05$) among treatments.

Characteristics of cauda epididymal Bali cattle spermatozoa

The characteristics of Bali cattle cauda epididymal spermatozoa observed in the present study included motility, concentration, viability, and abnormality (Table 1). These parameters are crucial for successful fertilization (Tanga et al., 2021).

Table 1. Characteristics of Bali cattle cauda epididymal spermatozoa aged 3-4-year-old

No	Parameter	Value
1.	Motility (%)	69.0 ± 0.02
2.	Concentration (x10^7/mL)	386.0 ± 19.61
3.	Viability (%)	86.1 ± 2.08
4.	Abnormality (%)	14.12 ± 2.13

According to the findings of Rombe et al. (2023), three-year-old Bali cattle demonstrated a higher spermatozoa concentration from all experimental groups before the application of natural dye treatment ($p < 0.05$) compared to younger (2-2.5 years old) bulls, indicating that age influences spermatozoa concentration. Motility is a common indicator of fertility in males (Santoso et al., 2021). The observed motility represented the overall motility value across all experimental groups before the application of natural dyes. The motility value in all experimental groups before the application of natural dye treatments was 69.0 ± 0.02%, slightly lower than that documented by Rombe et al. (2023), which was 71.2 ± 6.2%, and Wattimena et al. (2009), which was 75% ($p < 0.05$). This discrepancy may be due to differences in age, energy storage (ATP), and spermatogenesis maturity (James et al., 2020; Park and Pang, 2021).

Higher spermatozoa concentration correlates with higher conception rates (Keihani et al., 2021). The present study reported that the spermatozoa concentration of all experimental groups before the application of natural dye treatments was 386.0 ± 19.61 × 10^7/mL, which was higher than 310.1 ± 60.7 × 10^7/mL reported by Rombe et al. (2023; $p < 0.05$), likely due to differences in rearing systems and feeding (Kudratullah et al., 2024). Viability, indicating the percentage of live spermatozoa post-dilution (Komariah et al., 2023). In all experimental groups, before the application of natural dye treatment, the percentage of cells was recorded at 86.1 ± 2.08%, lower than the 95.9 ± 1.1% reported by Rombe et al. (2023; $p < 0.05$). These differences may result from variation in the collection time of the cauda epididymal and cattle type. Early collection post-slaughter yields better sperm quality (Sarsaifi et al., 2013; Harissatria et al., 2018; James et al., 2020). Live spermatozoa exhibited clear, unstained heads, whereas nonviable ones absorbed dye and appeared purple in all groups, consistent with Ducha (2012) and Ugur et al. (2019), who reported that compromised membrane permeability allows dye penetration. Spermatozoa abnormalities in all groups before the application of natural dye treatments were lower (14.12 ± 2.13%) than those reported by Rombe et al. (2023), at 20.6 ± 8.8% ($p < 0.05$). The low level of spermatozoa abnormalities may be due to reduced cold shock and osmotic imbalance, which cause structural damage (Gustina et al., 2023). Abnormalities can arise from primary factors, such as age, physical condition, and breed, or secondary factors, including handling and technical errors during laboratory procedures (Harissatria et al., 2018).

CONCLUSION

The combination of Giemsa with turmeric extract, beetroot extract, and areca nut extract was capable of identifying intact acrosomal caps in cauda epididymal spermatozoa of Bali cattle aged 3-4 years old. However, Giemsa combined with turmeric extract produced the most promising results, as reflected by the highest percentage of intact acrosomal caps (86.9 ± 2.0%) and clear staining outcomes. The Giemsa preparations exhibited a dark purple coloration, turmeric extract appeared yellowish, corresponding to the turmeric pigment, and areca nut extract appeared bluish, while beetroot extract demonstrated no detectable staining on the slides. Therefore, turmeric extract may serve as a practical, cost-effective, and environmentally friendly alternative for acrosome evaluation in veterinary reproductive laboratories, particularly in resource-limited settings. Further investigations are necessary to evaluate the effectiveness of turmeric extract in assessing acrosomal integrity in spermatozoa from different livestock species, as well as under different semen preservation conditions, including fresh, diluted, and cryopreserved samples. In addition, the biochemical interactions between turmeric pigments and sperm cell components should be explored to elucidate the underlying staining mechanism.

DECLARATIONS

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Authors' contributions

Angelina Novita Tethool, Yoppy Rivaldo Kafiar, and Andoyo Supriyantono conceived and designed the experiment. Angelina Novita Tethool and Yoppy Rivaldo Kafiar performed the laboratory experiments. Angelina Novita Tethool, Yoppy Rivaldo Kafiar, and Andoyo Supriyantono analyzed the data, interpreted the findings, drafted the manuscript, and revised the article. All authors have read and approved the final edition of the manuscript for submission to the journal.

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Ethical considerations

The authors affirmed that the present manuscript is their original work and has not been previously published. All authors have reviewed the content for plagiarism and verified that it reflects their own scientific findings.

Availability of data and materials

All data generated during this study are included in this article. Additional information is available from the authors upon reasonable request.

Competing interests

The authors declared no competing interests.

REFERENCES

Abu AH, Kisani AI, and Ahemen T (2016). Evaluation of sperm recovered after slaughter from cauda epidymides of red Sokoto bucks. Veterinary World, 9(12): 1440-1444. DOI: https/www.doi.org.10.14202/vetworld.2016.1440-1444

Alegbe EO and Uthman TO (2024). A review of history, properties, classification, applications and challenges of natural and synthetic dyes. Heliyon, 10(13): e33646. DOI: https://www.doi.org/10.1016/j.heliyon.2024.e33646

Amalraj A, Pius A, Gopi S, and Gopi S (2017). Biological activities of curcuminoids, other biomolecules from turmeric and their derivatives – A review. Journal of Traditional and Complementary Medicine, 7(2): 205-233. DOI: https://www.doi.org/10.1016/j.jtcme.2016.05.005

Ati NH, Rahayu P, Notosoedarmo S, and Limantara L (2010). The composition and the content of pigments from some dyeing plant for ikat weaving in Timorese regency, East Nusa Tenggara. Indonesian Journal of Chemistry, 6(3): 325-331. DOI: https://www.doi.org/10.22146/ijc.21741

Athala S (2021). Gastroprotective effectiveness of turmeric (*Curcuma domestica* Val) rhizome in aspirin induced stomach. Jurnal Ilmiah Kesehatan Sandi Husada, 10(2): 402-407. Available at: https://jurnalsandihusada.polsaka.ac.id/JIKSH/article/view/616/415

Baňas Š, Tvrdá E, Benko F, Ďuračka M, Čmiková N, Lukáč N, and Kačániová M (2024). Kaempferol as an alternative cryosupplement for bovine spermatozoa: Cytoprotective and membrane-stabilizing effects. International Journal of Molecular Sciences, 25(7): 4129. DOI: https://www.doi.org/10.3390/ijms25074129

Basha SJ, Kaur K, Kaur P, and Rehal J (2024). Curcuminoids: Composition, extraction, health benefits, delivery systems, and relation to COVID-19 treatment. Food Safety and Health, 2(1): 21-38. DOI: https://www.doi.org/10.1002/fsh3.12028

Bernecic NC, Donnellan E, O'Callaghan E, Kupisiewicz K, O'Meara C, Weldon K, Lonergan P, Kenny DA, and Fair S (2021). Comprehensive functional analysis reveals that acrosome integrity and viability are key variables distinguishing artificial insemination bulls of varying fertility. Journal of Dairy Science, 104(10): 1226-11241. DOI: https://www.doi.org/10.3168/jds.2021-20319

Chowdhury S, Das S, Gupta T, Sana D, and Bose S (2014). Evaluation of frozen semen by a crosomal integrity and sperm concentration - two vital quality parameters of male fertility in bovines. Exploratory Animal and Medical Research Animal Medical Research, 4(1): 101-107. Available at: https://www.cabidigitallibrary.org/doi/pdf/10.5555/20143302064

Ciuca MD and Racovita RC (2023). Curcunin: Overview of extraction methods, health benefits, and encapsulation and delivery using microemulsions and nanoemulsions. International Journal of Molecular Sciences, 24(10): 8874. DOI: https://www.doi.org/10.3390/ijms24108874

Cipriani S, Ricci E, Chiaffarino F, Esposito G, Dalmartello M, La Vecchia C, Negri E, and Parazzini F (2023). Trend of change of sperm count and concentration over the last two decades: A systematic review and meta-regression analysis. Andrology, 11(6): 997-1008. DOI: https://www.doi.org/10.1111/andr.13396

Ducha N, Susilawati T, Aulanniam, and Wahyuningsih S (2013). Motility and viability of Limousin bull sperm in CEP-2 extender with egg yolk supplementation during storage in refrigerator. Indonesian Journal of Veterinary Sciences, 7(1): 5-8. Available at: https://jurnal.usk.ac.id/JKH/article/view/555/464

Esteves SC, Sharma RK, Thomas Jr AJ, and Argawal A (2007). Evaluation of acrosomal status and sperm viability in fresh and cryopreserved specimens by the use of fluorescent peanut agglutinin lectin in conjunction with hypo-osmotic swelling test. International Brazil Journal of Urology, 33(3): 364-376. DOI: https://www.doi.org/10.1590/S1677-55382007000300009

Fannessia L, Karja N, Adnyane I, and Setiadi M (2015). Detection of acrosomal damage of ram spermatozoa during freezing process using lectin histhochemical technique. Jurnal Veteriner, 16(4): 560-568. DOI: https://dwww.doi.org/10.19087/jveteriner.2015.16.4.560

Gustina S, Hasbi, Sonjaya H, Baco S, Toleng AL, Mutmainna, and Farida S (2022). Motility and viability of spermatozoa of polled and horned Bali bull at each stage of the freezing process. Jurnal Ilmu dan Teknologi Peternakan Tropis, 9(1): 48-54. Available at: https://ojs.uho.ac.id/index.php/peternakan-tropis/article/view/18417

Gustina S, Maulidyah AMN, Jalil MFR, Bahmid NA, and Farida S (2023). The quality of fresh and frozen semen Bali bull assessed from different ejaculate volume. IOP Conference Series: Earth and Environmental Science, 1174(2023): 0120033. DOI: https://www.doi.org/10.1088/1755-1315/1174/1/012033

Harissatria, Hendri J, Jaswandi, and Hidayat F (2018). Evaluation of Cauda Epididymal Spermatozoa Quality of Simmental Crossbred Cattle at 5ºC Supplemented with Oviduct Fluid. Jurnal Peternakan, 15(2): 74-79. Available at: https://ejournal.uin-suska.ac.id/index.php/peternakan/article/view/5635/3394

Hartika G, Zulharmita, and Asra R (2021). Utilization of natural dyes substances for histological staining: A Review. Asian Journal of Pharmaceutical Research and Development, 9(1): 149-158. Available at: https://ajprd.com/index.php/journal/article/view/925

He Z, Xie M, Li QQ, Duan J, and Lu X (2022). Research progress on the microregulatory mechanisms of fertilization: A review. In Vivo, 36(5): 2002-2013. DOI: https://www.doi.org/10.21873/invivo.12926

Hirohashi N and Yanagimachi R (2018). Sperm acrosome reaction: Its site and role in fertilization. Biology of Reproduction, 99(1): 127-133. DOI: https://www.doi.org/10.1093/biolre/ioy045

James ER, Carrell DT, Aston KI, Jenkins TG, Yeste M, and Salas-Huetos A (2020). The role of the epididymis and the contribution of epididymosomes to mammalian reproduction. International Journal of Molecular Sciences, 21(15): 5377. DOI: https://www.doi.org/10.3390/ijms21155377

Keihani S, Verrilli LE, Zhang C, Presson AP, Hanson HA, Pastuszak AW, Johnstone EB, and Hotaling JM (2021). Semen parameter thresholds and time-to-conception in subfertile couples: How high is high enough?. Human Reproduction, 36(8): 2121-2133. DOI: https://www.doi.org/10.1093/humrep/deab133

Khan R, Azhar M, and Umair M (2024). Decoding the genes orchestrating egg and sperm fusion reactions and their roles in fertility. Biomedicines, 12(12): 2850. Available at: https://www.doi.org/10.3390/biomedicines12122850

Kudratullah, Arifiantini RI, Yuliani E, Pardede BP, Said S, and Purwantara B (2024). Semen characteristics, freezability, and application of motility-based protein markers (proAKAP4) in assessing the suitability of superior Bali bulls (*Bos sondaicus*) at the regional AI center. Reproduction and Breeding, 4(4): 279-286. Available at: https://www.sciencedirect.com/science/article/pii/S2667071224000450

Kumar A, Saxena A, and Anand M (2018). Evaluation of acrosomal integrity and viability in bull spermatozoa: comparison of cytochemical and fluorescent techniques. International Journal of Livestock Research, 9(2): 69-77. Available at: https://ijlr.org/ojs_journal/index.php/ijlr/article/view/1341

Kútvölgyi G, Stefler J, and Kovács A (2006). Viability and acrosome staining of stallion spermatozoa by Chicago sky blue and Giemsa. Biotechnic and Histochemistry, 81(4-6): 109-117. DOI: https://www.doi.org.10.1080/10520290600931007

Komariah, Zuhdi ML, Tahar MA, and Maulana T (2023). Frozen semen quality of simmental cattle in various commercial diluents. Jurnal Ilmu Produksi dan Teknologi Hasil Peternakan, 11(1): 41-47. DOI: https://www.doi.org/10.29244/jipthp.11.1.41-47

Leung ETY, Lee BKM, Lee Cheuk-Lun, Tian X, Lam KKW, Li RHW, Ng EHY, Yeung WSB, Ou Jian-Ping, and Chiu PCN (2023). The role of spermatozoa-zona pellucida interaction in selecting fertilization-competent spermatozoa in humans. Frontiers in Endocrinology, 20(14): 1135973. DOI: https://www.doi.org/10.3389/fendo.2023.1135973

Liliana C and Oana-Viorela N (2020). Red beetroot: Composition and health effects - A review. Journal of Nutritional Medicine and Diet Care, 6(1): 043. DOI: https://www.doi.org/10.23937/2572-3278.1510043

Manasa PSL, Kamble AD, and Chilakamarthi U (2023). Various extraction techniques of curcumin─A comprehensive review. ACS Omega, 8(38): 34868-34878. DOI: https://www.doi.org/10.1021/acsomega.3c04205

Mulia YS, Nurhayati D, Sulaeman, and Azhiima AF (2022). The effectiveness of beet and mangosteen skin as natural dye for Trichophyton rubrum fungus. Jurnal Riset Kesehatan Poltekkes Depkes Bandung, 14(2): 409-414. https://www.doi.org/10.34011/juriskesbdg.v14i2.2019

Nadgas SK, Smith L, Medina-Oertiz I, Hernandez-Encarnacion L, and Raychoudhury S (2016). Identification of bovine sperm acrosomal proteins that interact with a 32 kDa arcosomal matrix protein. Molecular and Cellular Biochemistry, 414(1-2): 153-169. DOI: https//www.doi.org/10.1007/s11010-016-2668-

Nemzer B, Pietrzkowski Z, Spórna A, Stalica P, Thresher W, Michałowski T, and Wybraniec S (2011). Betalainic and nutritional profiles of pigment-enriched red beet root (*Beta vulgaris* L.) dried extracts. Food Chemistry, 127(1): 42-53. DOI: https://www.doi.org/10.1016/j.foodchem.2010.12.081

Nirmala DS and Puspitasari RP (2017). The utilization of betel chewing ingredients (*Nginang*) as an alternative dye in microscopic slide of stem tissues. Berkala Ilmiah Pendidikan Biologi, 6(2): 111-117. Available at: https://ejournal.unesa.ac.id/index.php/bioedu/article/view/20849

Obeta U, Dajok G, Ejinaka O, Deko B, Yusuf BN, Ukpabi M, Amunum SG, and Achagh J (2022). Beetroot and turmeric as alternative dyes for haematoxylin an deosin in histological stainning. Scientific Research Journal of Clinical and Medical Sciences, 2(1): 54-58. DOI: https://www.doi.org/10.47310/srjcms.2022.v02i01.013

Park YJ and Pang MG (2021). Mitochondrial functionality in male fertility: From spermatogenesis to fertilization. Antioxidants, 10(1): 98. DOI: https://www.doi.org/10.3390/antiox10010098

Prihantoko KD, Yuliastuti F, Haniarti H, Kusumawati A, Widayati DT, and Budiyanto A (2020). The acrosome integrity examination of post-thawed spermatozoa of several ongole grade bull in Indonesia using giemsa staining method. IOP Conference Series: Earth and Environmental Science, 478: 012042. DOI: https://www.doi.org/10.1088/1755-1315/478/1/012042

Rautela R, Bisla A, Ngou AA, Kumar A, Ghosh SK, and Srivastava N (2020). Comparative efficacy of conventional stains for evaluation of plasmalemma and acrosome integrity of buffalo spermatozoa. Indian Journal of Animal Sciences, 90(20): 185-187. DOI: https://www.doi.org/10.56093/ijans.v90i2.98775

Santoso S, Herdis H, Arifiantini RI, Gunawan A, and Sumantri C (2021). Characteristics and potential production of frozen semen of Pasundan bull. Tropical Animal Science Journal, 44(1): 24-31. DOI: https://www.doi.org/10.5398/tasj.2021.44.1.24

Sarsaifi K, Rosnina Y, Ariff M, Wahid H, Hani H, Yimer N, Vejayan J, Win Naing S, and Abas M (2013). Effect of semen collection methods on the quality of pre- and post-thawed Bali cattle (*Bos javanicus*) spermatozoa. Reproduction in Domestic Animals, 48(6): 1006-1012. DOI: https://www.doi.org/10.1111/rda.12206

Sharan RN, Mehrotra R, Choudhury Y, and Asotra K (2012). Association of betel nut with carcinogenesis: Revisit with a clinical perspective. PLoS ONE, 7(8): e43759. DOI: https://www.doi.org/10.1371/journal.pone.0042759

Shankar SP, Mahadevan S, Karthika P, Arunachalam M, Satish MJ, and Chandamohan K (2022). A study of cytoplasmic staining in buccal smears using curucuma longa (turmeric) extract as an altenative to eosin as compared to H & E staining procedure. International Journal of Sciences, 6(S1): 3009-3015. DOI: https://www.doi.org/10.53730/ijhs.v6nS1.5127

Suhardi R, Megawati N, Ardhani F, Summpunn P, and Wuthisuthimethavee S (2020). Motility, viability, and abnormality of the spermatozoa of bali bull with andromed® and egg yolk-tris diluents stored at 4°C. Iranian Journal of Applied Animal Science, 10(2): 249-256. Available at: https://journals.iau.ir/article_673200_4988ec756516b6361f055f9a8311e47a.pdf

Sun W, Jiang S, Su J, Zhang J, Bao X, Ding R, Shi P, Li S, Wu C, Zhao G et al. (2020). The effects of cryopreservation on the acrosome structure, enzyme activity, motility, and fertility of bovine, ovine, and goat sperm. Animal Reproduction, 7(4): e20200219. DOI: https://www.doi.org/10.1590/1984-3143-AR2020-0219

Rombe IS, Purwaningsih, and Tethool AN (2023). Quality of Cauda Epididymis spermatozoa in Bali cattle based on different age. Jambura Journal of Animal Science, 5(2): 23-31. DOI: https://www.doi.org/10.35900/jjas.v5i2.19236

Tanga BM, Qamar Ay, Raza S, Bang S, Fang X, Yoon K, and Cho J (2021). Semen evaluation: Methodological advancements in sperm quality specific fertility assessment-A review. Animal Bioscience, 34(8): 1253-1270. DOI: https//www.doi.org/10.5713/ab.21.0072

Tethool AN, Ciptadi G, Wahjuningsih S, and Susilawati T (2022). Deterioration of frozen semen of Bali cattle after cooling at 5°C. World's Veterinary Journal, 12(4): 395-404. DOI: https://www.doi.org/10.54203/scil.2022.wvj50

Ugur MR, Saber AA, Evans HC, Gilmore AA, Hitit M, Arifiantini RI, Purwantara B, Kaya A, and Memili E (2019). Advances in cryopreservation of bull sperm. Frontiers in Veterinary Science, 27(6): 268. DOI: https://www.doi.org/10.3389/fvets.2019.00268

Wattimena J, Parera F, and Veerman M (2009). Effect of maltose concentration in Tris dilution on epididymal spermatozoa quality of Bali bull preserved at 5°C. Journal of the Indonesian Tropical Animal Agriculture, 34(4): 272-276. Available at: https://ejournal.undip.ac.id/index.php/jitaa/article/view/7593/6245