Research Article

Field Applicable Thawing Strategies for Frozen Bull Semen: Comparison of Warm-Water and Tap-Water Thawing on Post-Thaw Sperm Quality and Storability Across Cattle Breeds

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Abstract | This study compared the effects of thawing method and post-thaw storage duration on frozen semen quality across cattle breeds. A total of 240 semen straws from Limousin, Simmental, Holstein-Friesian (HF), Ongole, Bali, and Madura bulls were produced by the Singosari National Artificial Insemination Centre (SNAIC) using a tris-aminomethane extender. Semen was thawed in warm water (37 °C for 30 s) or tap water (28 °C for 45 s) and evaluated during 0–4 h of storage for motility, viability, abnormality, concentration, total motile spermatozoa (TMS), plasma membrane integrity (PMI), and intact acrosome cap (IAC). The experiment used a factorial randomized block design, and data were analysed by analysis of variance. Storage duration was the primary factor affecting sperm quality ($p < 0.05$). From 0-4 h, motility decreased from 46.92% to 21.88% (53.38%), viability from 60.33% to 37.97% (37.06%), PMI from 60.31% to 41.36% (31.43%), IAC from 59.48% to 38.97% (34.49%), and TMS from 13.09 to 5.96 million/straw (54.50%), whereas concentration and abnormality remained relatively stable. Thawing method had limited and sire-dependent effects, although warm-water thawing tended to better preserve some functional parameters. Among breeds, Limousin semen showed greater post-thaw resilience, whereas Bali semen deteriorated more rapidly. Therefore, frozen-thawed semen should be used as soon as possible, preferably within 2 h after thawing, with warm-water thawing offering a slight practical advantage.

Keywords | Frozen bull semen, Post-thaw sperm quality, Thawing method, Storage duration, Sperm functional integrity, Artificial insemination

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INTRODUCTION

Artificial insemination (AI) is a reproductive technology that improves cattle productivity, accelerates genetic improvement, and increases breeding efficiency (Tola and Deresa, 2025). Artificial insemination offers several advantages, including improved reproductive efficiency, reduced transmission of reproductive diseases, and

optimized use of genetically superior bulls (Ayantoye *et al.*, 2025; Cardoso Consentini *et al.*, 2021; Silva *et al.*, 2025). The success of AI depends on several factors, including the female's physiological condition, frozen semen quality, farm management practices, oestrus-detection accuracy, inseminator experience, and semen-handling protocols, particularly thawing procedures. Frozen semen is the primary biological material in AI programs because cryopreservation allows long-term storage and efficient distribution of genetic material in liquid nitrogen at −196 °C (Sharafi *et al.*, 2022). This process suppresses cellular metabolism and slows biological deterioration, thereby preserving sperm viability over extended periods. Nevertheless, cryopreservation exposes spermatozoa to structural and functional stress. During freezing and thawing, sperm cells may experience membrane disruption, osmotic imbalance, oxidative stress, and ice crystal formation, collectively decreasing motility, viability, and fertilizing capacity (Min *et al.*, 2024; Song *et al.*, 2026; Weldon *et al.*, 2026). Consequently, cryoinjury remains a significant limitation to the effectiveness of AI utilizing frozen semen.

Thawing is a critical phase in frozen semen management because it determines spermatozoa's ability to recover from cryoinjury and regain function. During this process, sperm cells are highly susceptible to thermal shock, osmotic stress, and ice recrystallization, all of which can compromise membrane stability and cellular function (Pezo *et al.*, 2024). Consequently, an appropriate thawing protocol is essential to preserve sperm survival and functional integrity after cryopreservation (Zenteno *et al.*, 2023). Previous studies have demonstrated that thawing temperature exerts a significant influence on post-thaw semen quality. Thawing at 37 °C for 30 s has been reported to enhance sperm motility, viability, and concentration in various cattle breeds, including Simmental and Bali cattle (Indriastuti *et al.*, 2020; Meles et al., 2022). In addition, thawing at 28 °C for 45 seconds has been shown to maintain sperm viability and functional characteristics (Fitriana et al., 2025). These findings collectively highlight the critical role of thawing conditions in determining post-thaw semen quality.

Frozen semen is typically thawed in warm water at a controlled temperature. Under field conditions, however, inseminators may also use tap water when warm water is unavailable. Most previous studies have evaluated thawing of frozen semen using warm-water methods at approximately 35–40 °C (Koch *et al.*, 2022; Zenteno *et al.*, 2023) and have reported that thawing at around 37 °C for 30 s is optimal for maintaining sperm motility and kinematic characteristics, as assessed by Computer-Assisted Sperm Analysis (Solís *et al.*, 2024). In contrast, this study used both conventional warm-water thawing and cold-water thawing. These methods differ in temperature stability, which may affect post-thaw outcomes. This issue is particularly relevant in Indonesia, where AI programs are often implemented under logistical constraints, especially in regions with limited access to liquid nitrogen and appropriate cryogenic facilities (Ratnawati *et al.*, 2023). The country's diverse geography, including mountainous areas and remote farming systems, further complicates maintaining optimal semen handling during field service (Taufiq *et al.*, 2025).

In many cases, inseminators must travel considerable distances to reach farms, and transporting liquid nitrogen containers is not always practical. Under such conditions, thawed semen may need to be kept temporarily under non-cryogenic conditions before insemination, increasing the risk of sperm quality deterioration (Zhang *et al.*, 2021). Therefore, evaluating practical thawing alternatives and post-thaw storability is important for improving AI outcomes under field conditions. The response of spermatozoa to thawing and subsequent storage may vary among bulls of different cattle breeds. Such variation may be associated with differences in genetic background, sperm membrane lipid composition, metabolism, and inherent cryotolerance, which may influence susceptibility to cryodamage and post-thaw survival. This issue mostly happens in Indonesia, where AI is widely used in genetically diverse cattle populations, including Limousin, Simmental, Holstein-Friesian (HF), Ongole, Bali, and Madura. However, previous studies investigating thawing protocols have largely focused on a limited range of breeds and controlled laboratory settings, while evidence on breed-specific responses to thawing under field-relevant conditions remains limited (Indriastuti *et al.*, 2020; Koch *et al.*, 2022; Solís *et al.*, 2024; Zenteno *et al.*, 2023). Comparative information on warm-water and tap-water thawing, including post-thaw semen storability, remains limited, particularly across different cattle breeds. This gap is important because field-applicable thawing methods must be both biologically effective and operationally practical. Improving semen-handling practices and the efficiency of AI programs also supports the achievement of the Sustainable Development Goals (SDGs). More reliable AI outcomes can enhance cattle productivity and farmers' livelihoods, thereby contributing to SDG 1 (No Poverty), while improving the availability and sustainability of animal-source food production supports SDG 2 (Zero Hunger). Furthermore, optimizing the use of frozen semen, reducing semen losses caused by inappropriate thawing and storage, and promoting efficient use of superior genetic resources are consistent with SDG 12 (Responsible Consumption and Production). Therefore, this study aimed to compare the effects of warm-water and tap-water thawing on post-thaw sperm quality and storability in frozen semen from bulls of different breeds. Sperm quality

was evaluated during 0–4 h of post-thaw storage based on motility, viability, abnormality, concentration, total motile spermatozoa (TMS), plasma membrane integrity (PMI), and intact acrosome cap (IAC). The findings are expected to provide practical evidence to improve semen-handling recommendations and support more efficient AI programs under field conditions especially in tropical countries such as Indonesia.

MATERIALS AND METHODS

Animals and semen source

Frozen semen used in this study was obtained from six bulls representing different cattle breeds: Limousin (Macedonia; ID 816119), Simmental (Nighthawk; ID 617118), HF (Diplomasi; ID 317119), Ongole (Jango; ID 21739), Bali (Sabala; ID 116132), and Madura (Lombang; ID 161033). The bulls were 7-15 years old and weighed 508-986 kg. All bulls were maintained at the Singosari National Artificial Insemination Centre (SNAIC), Malang, Indonesia, and had passed breeding bull soundness evaluation before semen collection. Semen was routinely collected twice weekly using an artificial vagina by trained technicians. Only ejaculates with individual motility ≥ 70% and sperm abnormality < 20% were processed for frozen semen production. All semen straws used in this study were prepared using a tris-aminomethane extender.

Experimental design and thawing procedures

Semen from six bulls, representing six different cattle breeds (one bull per breed), was subjected to two thawing treatments: warm-water thawing at 37 °C for 30 s (T0) and tap-water thawing at 28 °C for 45 s (T1). The factorial experiment included 10 replicates for each bull × thawing treatment combination × storage duration, with two straws (0.25 mL per straw) used per replicate, resulting in a total of 240 frozen semen straws (6 bulls × 2 treatments × 10 replicates × 2 straws). Following thawing, the semen was transferred into microtube (1.5 mL) and maintained at the same temperature as the respective thawing treatment throughout the storage period. Post-thaw semen quality was evaluated repeatedly at five storage times: 0, 1, 2, 3, and 4 h. The evaluated parameters included sperm motility, viability, abnormality, concentration, TMS, PMI, and IAC.

Post thaw semen evaluation

Sperm motility

Individual motility was assessed under a light microscope at 400 × magnification. A 5 µL semen sample was placed on a glass slide, covered with a cover slip, and evaluated in five different microscopic fields. The mean percentage of progressively motile spermatozoa was recorded (Rahmawati *et al.*, 2025).

Sperm viability

Sperm viability was assessed using eosin–nigrosine staining according to Asadpour *et al.* (2021). Briefly, 5 µL of semen was mixed with 5 µL of eosin-nigrosine stain on a glass slide, smeared using another slide at a 45° angle, air-dried, and examined under a light microscope at 400 × magnification. At least 200 spermatozoa were counted. Live spermatozoa remained unstained, whereas dead spermatozoa absorbed the stain. Viability was calculated as the proportion of live spermatozoa out of the total spermatozoa counted, expressed as a percentage.

Sperm abnormality

Abnormality was assessed on eosin–nigrosine smears prepared as described for viability. A minimum of 200 spermatozoa were examined under a light microscope at 400 × magnification. Morphological defects included abnormalities of the head and tail, such as microcephalic or macrocephalic heads, detached or broken heads, and coiled or detached tails (Arora *et al.*, 2024). Abnormality was expressed as the percentage of abnormal sperm out of the total sperm counted.

Sperm concentration

Sperm concentration was determined using a haemocytometer. Briefly, 10 µL of semen was diluted in 990 µL of 3% NaCl, homogenized, and loaded into the counting chamber. Sperm were counted in five squares under a light microscope at 400 × magnification. Concentration was calculated according to Utami *et al.* (2026) as:

$$\text{Sperm concentration (million/0.25 mL)} = N \times 5 \times DF \times 0.25 \times 10{,}000$$

Where; N is the average sperm count, DF is the dilution factor (1:100), 0.25 is the straw volume (mL), and 10,000 is the haemocytometer correction factor.

Total motile spermatozoa

TMS was calculated according to Wahyudi *et al.* (2025) as the product of sperm concentration (million/straw) and the percentage of progressive motility, representing the total number of progressively motile spermatozoa per straw.

Plasma membrane integrity

PMI was evaluated using the hypoosmotic swelling test (HOST) as described by Pardede *et al.* (2020). 10 µL of semen was added to 1000 µL of hypoosmotic solution and incubated in a water bath at 37 °C for 30 min. After incubation, 10 µL of the sample was placed on an object glass and covered with a cover glass. The sample was evaluated under a light microscope at 400 × magnification, and at least 200 spermatozoa were evaluated. Spermatozoa exhibiting curled or coiled tails were considered to have intact plasma membranes. PMI was determined as the

percentage of spermatozoa with curled or coiled tails relative to the total number of spermatozoa evaluated.

Intact acrosome cap

An IAC was evaluated as describe by Pramudhita *et al.* (2026). Briefly, 25 µL of semen was added to 100 µL of NaCl solution containing 1% formalin, followed by gentle homogenization for 5 min using a figure-eight motion. Subsequently, a thin smear of the mixture was prepared on a glass slide, and a minimum of 200 spermatozoa were evaluated. IAC was determined as the percentage of spermatozoa exhibiting an intact acrosomal cap relative to the total number of spermatozoa evaluated.

Statistical analysis

The data obtained were tabulated and statistically analysed using R (version 4.4.3; R core team, Vienna, Austria). The experiment was arranged as a factorial experiment in a randomized block design (RBD), with thawing method as factor A and post-thaw storage duration (0, 1, 2, 3, and 4 h) as factor B. Each treatment combination consisted of 10 replicates, with replicate treated as the blocking factor. Data were analysed using analysis of variance (ANOVA) to evaluate the main effects of thawing method and post-thaw storage duration, as well as their interaction, on semen quality parameters. When significant differences were detected ($p < 0.05$), mean comparisons were performed using Duncan's multiple range test (DMRT). Each breed was represented by a single bull; the effect of bull was interpreted cautiously as variation among individuals representing different breeds rather than as pure breed effects.

RESULTS

Post-thaw sperm motility

Post-thaw sperm motility decreased significantly with increasing storage duration across all six cattle breeds under both thawing treatments (Table 1). At 0 h, initial motility ranged from 45.30 ± 2.63% to 45.30 ± 2.63%. Motility remained relatively stable during the first 1–2 h, but declined dramatically thereafter, particularly at 4 h. Storage duration was the primary factor contributing to the reduction in sperm motility, whereas the effect of thawing method was relatively small and varied among breeds. Warm-water thawing tended to maintain higher motility during prolonged post-thaw storage in Limousin, Simmental, and Ongole semen. In contrast, 4 h of post-thaw storage resulted in notable decline in motility. Based on the observations across the six cattle breeds, Limousin semen demonstrated a greater ability to maintain post-thaw quality, whereas Bali semen exhibited lower tolerance to prolonged post-thaw storage.

Post-thaw sperm viability

Post-thaw sperm viability declined markedly with increasing storage duration across all six cattle breeds subjected to two different thawing methods (Table 2). At 0 h of storage, viability ranged from 58.72% to 63.45%, with the highest value observed in Limousin semen under the T0 treatment. The other breeds showed relatively similar initial viability, indicating that spermatozoa were able to maintain good survival immediately after thawing. Viability gradually decreased during storage, with a more pronounced decline observed at 3–4 h. At 4 h, Limousin semen was

Table 1: Post-thaw sperm motility of different cattle breeds under two thawing methods during storage.

Breed	Treatments	Sperm motility (%)				
		0 h	1 h	2 h	3 h	4 h
Limousin	T0	49.00 ± 2.00^{a}	46.50 ± 2.29^{ab}	43.50 ± 2.29^{ab}	40.50 ± 2.69^{b}	33.40 ± 5.02^{c}
	T1	48.50 ± 2.29^{a}	47.00 ± 2.45^{ab}	42.00 ± 2.45^{ab}	40.00 ± 2.24^{b}	29.00 ± 5.83^{c}
Simmental	T0	48.00 ± 2.60^{a}	47.50 ± 3.50^{a}	44.00 ± 2.10^{a}	37.50 ± 3.50^{a}	26.00 ± 10.70^{b}
	T1	46.50 ± 2.40^{a}	44.50 ± 2.80^{a}	43.50 ± 2.40^{a}	38.50 ± 2.40^{a}	25.00 ± 9.10^{b}
HF	T0	46.00 ± 1.76^{a}	45.90 ± 1.73^{a}	42.50 ± 2.32^{a}	36.30 ± 8.86^{a}	17.90 ± 9.46^{b}
	T1	45.30 ± 2.63^{a}	45.00 ± 2.16^{a}	43.40 ± 2.50^{a}	39.00 ± 2.91^{a}	24.70 ± 9.46^{b}
Ongole	T0	48.50 ± 2.29^{a}	45.50 ± 3.40^{a}	44.00 ± 2.00^{a}	40.50 ± 2.69^{a}	18.00 ± 7.14^{b}
	T1	46.50 ± 3.20^{a}	46.00 ± 2.00^{a}	43.50 ± 2.29^{ab}	38.00 ± 4.00^{b}	15.50 ± 5.68^{c}
Bali	T0	46.50 ± 3.37^{a}	46.00 ± 3.16^{a}	43.50 ± 3.37^{a}	37.50 ± 2.64^{a}	12.50 ± 7.55^{b}
	T1	45.50 ± 3.69^{a}	44.50 ± 2.84^{a}	41.00 ± 2.11^{a}	37.50 ± 2.64^{a}	13.00 ± 7.15^{b}
Madura	T0	46.90 ± 0.70^{a}	46.10 ± 1.79^{a}	43.60 ± 1.43^{b}	38.10 ± 3.35^{bc}	22.60 ± 5.87^{c}
	T1	45.90 ± 1.37^{a}	45.70 ± 1.57^{a}	43.20 ± 1.03^{ab}	36.80 ± 3.33^{b}	24.90 ± 6.08^{b}

Values are presented as mean ± standard deviation (SD). Different superscript letters (a–c) within the same row indicate significant differences among storage times (0–4 h) within each breed and treatment ($p < 0.05$) according to Duncan's multiple range test (DMRT). T0 = thawing at 37 °C for 30 s; T1 = thawing at 28 °C for 45 s. HF = Holstein-Friesian; Ongole.

Table 2: Post-thaw sperm viability of different cattle breeds under two thawing methods during storage.

Breed	Treatments	Sperm viability (%)				
		0 h	1 h	2 h	3 h	4 h
Limousin	T0	63.45 ± 1.84^{a}	61.12 ± 1.89^{ab}	57.81 ± 4.57^{ab}	53.81 ± 4.21^{bc}	48.14 ± 5.67^{c}
	T1	60.77 ± 2.63^{a}	59.55 ± 1.92^{a}	57.38 ± 1.84^{ab}	52.44 ± 3.67^{b}	45.52 ± 4.51^{c}
Simmental	T0	59.90 ± 3.91^{a}	58.18 ± 3.84^{ab}	57.44 ± 4.77^{ab}	50.33 ± 1.54^{b}	39.24 ± 6.17^{c}
	T1	59.01 ± 3.71^{a}	57.67 ± 3.34^{a}	56.49 ± 4.81^{a}	50.27 ± 2.63^{a}	38.09 ± 8.59^{b}
HF	T0	60.61 ± 1.51^{a}	59.13 ± 3.02^{a}	57.25 ± 5.07^{a}	49.46 ± 14.1^{ab}	34.27 ± 6.78^{b}
	T1	59.71 ± 2.66^{a}	59.49 ± 2.17^{a}	57.61 ± 2.81^{a}	52.78 ± 5.19^{a}	39.70 ± 6.30^{b}
Ongole	T0	60.26 ± 3.04^{a}	59.49 ± 3.11^{a}	57.26 ± 3.28^{a}	52.27 ± 2.46^{a}	34.24 ± 8.96^{b}
	T1	59.72 ± 4.22^{a}	58.89 ± 4.12^{a}	55.66 ± 3.43^{a}	51.08 ± 3.30^{a}	32.12 ± 5.52^{b}
Bali	T0	60.23 ± 2.53^{a}	57.76 ± 3.21^{a}	56.61 ± 2.16^{ab}	48.38 ± 4.13^{b}	32.31 ± 7.23^{c}
	T1	58.72 ± 1.76^{a}	57.41 ± 2.06^{a}	55.12 ± 2.21^{ab}	48.84 ± 5.03^{b}	30.48 ± 6.99^{c}
Madura	T0	60.15 ± 2.74^{a}	60.68 ± 2.06^{a}	59.02 ± 2.14^{a}	54.94 ± 3.77^{a}	39.45 ± 7.41^{b}
	T1	61.39 ± 1.42^{a}	60.15 ± 3.13^{ab}	58.36 ± 2.89^{ab}	53.90 ± 3.15^{b}	42.11 ± 4.58^{c}

Values are presented as mean ± standard deviation (SD). Different superscript letters (a–c) within the same row indicate significant differences among storage times (0–4 h) within each breed and treatment ($P < 0.05$) according to Duncan's multiple range test (DMRT). T0 = thawing at 37 °C for 30 s; T1 = thawing at 28 °C for 45 s; HF = Holstein-Friesian; PO = Ongole.

Table 3: Post-thaw sperm abnormality of different cattle breeds under two thawing methods during storage.

Breed	Treatments	Sperm abnormality (%)				
		0 h	1 h	2 h	3 h	4 h
Limousin	T0	5.28 ± 0.77	4.51 ± 0.77	5.39 ± 0.87	4.78 ± 1.08	4.32 ± 1.24
	T1	5.36 ± 0.97	5.60 ± 0.82	4.76 ± 0.62	5.30 ± 1.13	4.89 ± 0.60
Simmental	T0	5.26 ± 0.30	5.25 ± 0.51	5.34 ± 0.30	5.10 ± 0.25	5.40 ± 0.50
	T1	5.63 ± 0.62	5.24 ± 0.37	5.69 ± 0.83	5.47 ± 0.47	5.68 ± 0.45
HF	T0	5.33 ± 1.55	4.92 ± 1.23	5.13 ± 0.91	5.04 ± 1.00	5.08 ± 0.81
	T1	5.66 ± 1.44	5.43 ± 1.54	5.28 ± 0.99	4.91 ± 1.25	5.46 ± 0.93
PO	T0	5.62 ± 1.20	5.05 ± 1.04	4.32 ± 0.41	4.97 ± 0.68	5.46 ± 1.16
	T1	4.70 ± 0.59	4.96 ± 0.80	4.84 ± 0.81	5.20 ± 1.04	5.52 ± 1.13
Bali	T0	5.14 ± 0.83	5.00 ± 0.95	6.03 ± 0.93	5.14 ± 0.60	5.83 ± 1.28
	T1	4.95 ± 1.49	5.28 ± 0.65	5.32 ± 1.28	6.08 ± 1.69	5.40 ± 0.90
Madura	T0	5.61 ± 1.69	5.03 ± 0.86	5.45 ± 1.13	5.07 ± 0.90	6.31 ± 1.37
	T1	5.30 ± 1.41	4.98 ± 0.82	5.50 ± 0.93	5.67 ± 1.84	5.35 ± 1.00

Values are presented as mean ± standard deviation (SD). 0–4 h = different storage times. T0 = thawing at 37 °C for 30 s; T1 = thawing at 28 °C for 45 s; HF = Holstein-Friesian; Ongole.

still able to maintain viability at 48.14 ± 5.67% (T0) and 45.52 ± 4.51% (T1), whereas Ongole, Simmental, Bali, HF, and Madura semen showed lower values with varying rates of decline. Overall, all cattle breeds exhibited a consistent decrease in sperm viability, although the rate of decline differed among breeds. Warm-water thawing generally maintained slightly higher sperm viability than tap-water thawing (T1).

Post-thaw sperm abnormality

Post-thaw sperm abnormality remained low and relatively stable during storage across all cattle breeds and under both thawing methods (Table 3). Across the 0–4 h post-thaw period, abnormality values ranged from 4.32% to 6.31%, suggesting minimal morphological damage during storage. At the end of storage, Limousin semen exhibited lower abnormality than Ongole semen, with values of 4.32 ± 1.24% and 4.89 ± 0.60% in Limousin under T0 and T1, respectively, compared with 5.46 ± 1.16% and 5.52 ± 1.13% in Ongole semen. Simmental and Bali semen showed broadly similar trends, although Bali tended to have higher abnormality at later storage times, particularly at 2 h under T0 (6.03 ± 0.93%) and 3 h under T1 (6.08 ± 1.69%). HF and Madura semen also exhibited relatively stable abnormality profiles, with Madura showing a numerically higher value than HF at 4 h under T0.

Table 4: Post-thaw sperm concentration of different cattle breeds under two thawing methods during storage.

Breed	Treatments	Sperm concentration (million/straw)				
		0 h	1 h	2 h	3 h	4 h
Limousin	T0	25.13 ± 6.16	25.13 ± 6.16	25.13 ± 6.16	25.13 ± 6.16	25.13 ± 6.16
	T1	25.82 ± 6.44	25.82 ± 6.44	25.82 ± 6.44	25.82 ± 6.44	25.82 ± 6.44
Simmental	T0	28.88 ± 6.30	28.88 ± 6.30	28.88 ± 6.30	28.88 ± 6.30	28.88 ± 6.30
	T1	26.69 ± 3.92	26.69 ± 3.92	26.69 ± 3.92	26.69 ± 3.92	26.69 ± 3.92
HF	T0	27.19 ± 6.43	27.19 ± 6.43	27.19 ± 6.43	27.19 ± 6.43	27.19 ± 6.43
	T1	28.19 ± 6.84	28.19 ± 6.84	28.19 ± 6.84	28.19 ± 6.84	28.19 ± 6.84
Ongole	T0	34.19 ± 6.89	34.19 ± 6.89	34.19 ± 6.89	34.19 ± 6.89	34.19 ± 6.89
	T1	38.50 ± 6.85	38.50 ± 6.85	38.50 ± 6.85	38.50 ± 6.85	38.50 ± 6.85
Bali	T0	25.80 ± 4.16	25.80 ± 4.16	25.80 ± 4.16	25.80 ± 4.16	25.80 ± 4.16
	T1	27.40 ± 5.88	27.40 ± 5.88	27.40 ± 5.88	27.40 ± 5.88	27.40 ± 5.88
Madura	T0	27.56 ± 5.69	27.56 ± 5.69	27.56 ± 5.69	27.56 ± 5.69	27.56 ± 5.69
	T1	25.63 ± 4.43	25.63 ± 4.43	25.63 ± 4.43	25.63 ± 4.43	25.63 ± 4.43

Values are presented as mean ± standard deviation (SD). 0–4 h = storage time. T0 = thawing at 37 °C for 30 s; T1= thawing at 28 °C for 45 s; HF= Holstein-Friesian; PO= Ongole.

Post-thaw sperm concentration

Sperm concentration (million/straw ± SD) remained constant across post-thaw storage times (0–4 h) within each breed and thawing treatment (Table 4). In Limousin semen, the concentration was 25.13 ± 6.16 million/straw under T0 and 25.82 ± 6.44 million/straw under T1, whereas the concentration values in Ongole semen were 34.19 ± 6.89 and 38.50 ± 6.85 million/straw, respectively. Similarly stable values were recorded under T0 and T1, respectively, in Simmental semen (28.88 ± 6.30 and 26.69 ± 3.92 million/straw), Bali semen (25.80 ± 4.16 and 27.40 ± 5.88 million/straw), HF semen (27.19 ± 6.43 and 28.19 ± 6.84 million/straw), and Madura (27.56 ± 5.69 and 25.63 ± 4.43 million/straw). Across all breed-treatment combinations, sperm concentration ranged from 25.13 ± 6.16 to 38.50 ± 6.85 million/straw, with the highest value observed in Ongole semen under T1 and the lowest in Limousin semen under T0. Overall, these findings indicate that sperm concentration remained unchanged throughout the 4 h post-thaw storage period within each breed and treatment, although breed-related variation was evident. Limousin and PO semen differed significantly in sperm concentration ($p < 0.01$), with Ongole semen consistently containing more spermatozoa per straw than Limousin semen. In contrast, Simmental and Bali semen did not differ significantly under either treatment ($p > 0.05$), although Simmental tended to show numerically higher concentrations. HF and Madura semen also remained within a comparable range.

Post-thaw total motile spermatozoa

TMS calculated as individual motility multiplied by sperm concentration, represents the number of progressively motile spermatozoa per straw and serves as an important indicator of the fertilizing potential of frozen–thawed semen. In this study, TMS declined steadily as post-thaw storage time increased across all breeds and under both thawing treatments (Table 5). At 0 h, TMS ranged from 11.77 ± 2.11 to 17.57 ± 3.24 million/straw, with Ongole semen under T1 showing the highest value. By 4 h, TMS had decreased markedly in all breeds, reaching 7.62 ± 1.49 and 7.47 ± 2.05 million/straw in Limousin, 7.66 ± 4.16 and 6.61 ± 2.77 million/straw in Simmental, 4.57 ± 1.95 and 6.59 ± 2.14 million/straw in HF, 5.22 ± 2.33 and 6.45 ± 2.12 million/straw in Ongole, 3.10 ± 1.73 and 3.44 ± 1.72 million/straw in Bali, and 6.33 ± 2.42 and 6.44 ± 2.13 million/straw in Madura semen under T0 and T1, respectively. Ongole semen had the highest initial TMS, whereas Bali and Madura often showed the lowest values. Over prolonged storage, Simmental maintained higher TMS than Bali, indicating better post-thaw endurance, and Madura generally retained slightly higher values than HF at 4 h.

Post-thaw plasma membrane integrity

Post-thaw PMI declined progressively as post-thaw storage time increased across all breeds. It was significantly affected by storage duration and cattle breed, whereas the thawing method had no significant effect (Table 6). Initial values were relatively similar among breed–treatment combinations, ranging from 58.98 ± 2.92% to 61.37 ± 1.82%, but declined markedly by 4 h to 34.26 ± 5.43% – 49.87 ± 4.40%. The pattern of decline differed among breeds: PO, Bali, and Madura semen remained relatively stable up to 3 h, whereas Limousin, Simmental, and HF semen were generally stable only up to 2 h and began to decline at 3 h. By 4 h, all breed–treatment combinations showed a marked reduction in PMI, with Limousin retaining the highest values and Bali the lowest.

Table 5: Total motile spermatozoa of different cattle breed under two thawing methods during storage.

Breed	Treatments	Total motile spermatozoa (million/straw)				
		0 h	1 h	2 h	3 h	4 h
Limousin	T0	11.86 ± 2.85	11.44 ± 2.72	10.72 ± 2.69	9.75 ± 2.07	7.62 ± 1.49
	T1	11.97 ± 2.96	11.67 ± 2.62	10.88 ± 2.87	10.08 ± 2.67	7.47 ± 2.05
Simmental	T0	13.51 ± 3.03	13.30 ± 2.94	12.81 ± 2.68	11.01 ± 2.94	7.66 ± 4.16
	T1	12.24 ± 1.91^{a}	11.93 ± 2.00^{a}	11.50 ± 1.86^{a}	10.09 ± 1.41^{ab}	6.61 ± 2.77^{b}
HF	T0	12.53 ± 3.13^{a}	12.47 ± 2.96^{a}	11.49 ± 2.46^{a}	10.10 ± 3.72^{ab}	4.57 ± 1.95^{b}
	T1	12.83 ± 3.52	12.66 ± 3.12	12.24 ± 3.15	11.09 ± 3.24	6.59 ± 2.14
Ongole	T0	15.77 ± 3.29^{a}	15.38 ± 3.51^{a}	14.73 ± 3.31^{a}	13.43 ± 3.28^{a}	5.22 ± 2.33^{b}
	T1	17.57 ± 3.24^{a}	17.31 ± 3.26^{a}	16.38 ± 3.36^{a}	14.75 ± 3.67^{a}	6.45 ± 2.12^{b}
Bali	T0	11.81 ± 2.12^{a}	11.51 ± 2.17^{a}	10.84 ± 1.78^{a}	9.25 ± 1.54^{a}	3.10 ± 1.73^{b}
	T1	12.35 ± 3.10^{a}	12.25 ± 2.99^{a}	11.24 ± 2.52^{a}	10.02 ± 1.97^{a}	3.44 ± 1.72^{b}
Madura	T0	12.92 ± 2.64^{a}	12.70 ± 2.60^{a}	12.00 ± 2.46^{a}	10.53 ± 2.55^{ab}	6.33 ± 2.42^{b}
	T1	11.77 ± 2.11^{a}	11.72 ± 2.17^{a}	11.07 ± 1.97^{a}	9.51 ± 2.27^{ab}	6.44 ± 2.13^{b}

Values are presented as mean ± standard deviation (SD). Different superscript letters (a–c) within the same row indicate significant differences among storage times (0–4 h) within each breed and treatment ($p < 0.05$) according to Duncan's multiple range test (DMRT). T0 = thawing at 37°C for 30 s; T1 = thawing at 28 °C for 45 s; HF = Holstein-Friesian; Ongole.

Table 6: Plasma membrane integrity of different cattle breeds under two thawing methods during storage.

Breed	Treatments	Plasma membrane integrity (%)				
		0 h	1 h	2 h	3 h	4 h
Limousin	T0	60.68 ± 2.09^{a}	59.90 ± 2.47^{a}	58.85 ± 4.89^{a}	55.54 ± 4.82^{ab}	49.87 ± 4.40^{b}
	T1	60.30 ± 4.04^{a}	59.33 ± 2.45^{a}	56.37 ± 5.76^{ab}	54.59 ± 4.75^{ab}	47.77 ± 3.77^{b}
Simmental	T0	59.62 ± 2.41^{a}	59.46 ± 3.25^{a}	59.11 ± 3.36^{a}	53.07 ± 1.55^{b}	44.03 ± 5.40^{c}
	T1	58.98 ± 2.92^{a}	58.98 ± 3.00^{a}	59.27 ± 3.74^{a}	53.16 ± 3.24^{a}	41.01 ± 7.37^{b}
HF	T0	60.53 ± 3.82^{a}	61.26 ± 2.52^{a}	59.70 ± 3.30^{a}	54.78 ± 8.90^{ab}	37.18 ± 7.15^{b}
	T1	61.07 ± 1.76	60.08 ± 3.01	59.02 ± 4.25	55.65 ± 5.06	42.35 ± 5.95
Ongole	T0	60.37 ± 2.23^{a}	60.76 ± 2.60^{a}	59.52 ± 2.89^{a}	55.97 ± 2.21^{a}	39.03 ± 7.68^{b}
	T1	60.86 ± 2.46^{a}	59.95 ± 2.44^{a}	58.69 ± 2.78^{a}	55.10 ± 2.92^{a}	35.39 ± 6.06^{b}
Bali	T0	60.21 ± 5.04^{a}	58.36 ± 4.10^{a}	58.15 ± 2.19^{a}	52.98 ± 4.99^{a}	34.97 ± 5.31^{b}
	T1	59.04 ± 3.81^{a}	57.78 ± 3.37^{a}	57.31 ± 1.63^{a}	51.05 ± 4.99^{a}	34.26 ± 5.43^{b}
Madura	T0	61.37 ± 1.82^{a}	61.25 ± 1.72^{a}	60.00 ± 2.11^{a}	57.97 ± 3.18^{ab}	43.68 ± 8.56^{b}
	T1	60.72 ± 1.92^{a}	60.15 ± 2.94^{a}	59.35 ± 2.92^{a}	57.35 ± 3.38^{ab}	46.73 ± 4.70^{b}

Values are presented as mean ± standard deviation (SD). Different superscript letters (a–c) within the same row indicate significant differences among storage times (0–4 h) within each breed and treatment ($p < 0.05$) according to Duncan's multiple range test (DMRT). T0 = thawing at 37°C for 30 s; T1 = thawing at 28 °C for 45 s; HF = Holstein-Friesian; Ongole.

Post-thaw intact acrosome cap

The proportion of spermatozoa with an IAC declined progressively with increasing post-thaw storage time in all breeds and under both thawing treatments (Table 7). Initial values ranged from 58.23 ± 1.99% to 60.79 ± 1.99%, then decreased markedly by 4 h to approximately 33.02 ± 6.86% – 47.09 ± 5.10%. The pattern of decline varied among breeds, indicating an interaction between storage duration and breed. Overall, Limousin and Bali semen showed a more pronounced decline after 2–3 h of post-thaw storage. Simmental semen began to decrease after 3 h of storage, whereas HF and Ongole semen exhibited relatively greater tolerance to prolonged storage. Madura semen remained relatively stable for up to 3 h, followed by a marked decline at 4 h. In addition, the thawing method did not cause significant differences under the conditions of this study.

DISCUSSION

AI programs for cattle depend on frozen semen stored in liquid nitrogen to maintain quality. In practice, however, these programs face major logistical constraints limited access to liquid nitrogen in remote areas (Ratnawati *et al.*, 2023), long distances between farms and inseminators, and restricted AI service coverage (Mahbubiy *et al.*, 2026; Taufiq *et al.*, 2025). In Indonesia, the widespread distribution of farms further complicates semen distribution and reduces the likelihood of performing insemination at the optimal time during the short oestrus period (Fitriana et al., 2025).

Table 7: Intact acrosome cap of different cattle breeds under two thawing methods during storage.

Breed	Treatments	Intact acrosome cap (%)				
		0 h	1 h	2 h	3 h	4 h
Limousin	T0	60.27 ± 1.76^{a}	59.26 ± 1.13^{a}	57.14 ± 1.31^{ab}	52.99 ± 2.84^{bc}	47.09 ± 5.10^{c}
	T1	60.24 ± 1.18^{a}	58.36 ± 1.30^{ab}	56.60 ± 0.87^{ab}	51.87 ± 3.77^{bc}	46.40 ± 5.60^{c}
Simmental	T0	58.97 ± 2.09^{a}	56.94 ± 2.89^{ab}	55.46 ± 2.17^{ab}	51.10 ± 3.62^{b}	39.75 ± 6.19^{c}
	T1	58.23 ± 1.99^{a}	56.52 ± 2.67^{a}	54.67 ± 2.73^{a}	51.13 ± 4.15^{ab}	42.14 ± 10.53^{c}
HF	T0	59.70 ± 1.51^{a}	58.23 ± 1.80^{a}	56.17 ± 2.70^{a}	51.38 ± 6.38^{a}	35.33 ± 5.38^{b}
	T1	59.31 ± 1.40^{a}	58.93 ± 1.73^{a}	57.36 ± 1.88^{a}	51.74 ± 6.15^{a}	40.00 ± 6.72^{b}
Ongole	T0	59.40 ± 1.57^{a}	58.91 ± 2.33^{a}	56.32 ± 2.44^{a}	53.58 ± 1.03^{a}	36.56 ± 7.42^{b}
	T1	59.07 ± 2.58^{a}	57.87 ± 2.14^{a}	55.27 ± 2.15^{a}	52.40 ± 2.26^{a}	33.47 ± 5.14^{b}
Bali	T0	59.29 ± 1.21^{a}	57.55 ± 1.52^{a}	56.12 ± 1.68^{ab}	49.35 ± 3.29^{b}	33.02 ± 6.86^{c}
	T1	58.50 ± 0.55^{a}	57.28 ± 1.73^{a}	55.45 ± 2.04^{a}	47.58 ± 3.65^{b}	33.03 ± 6.83^{c}
Madura	T0	60.79 ± 1.99^{a}	59.56 ± 1.37^{a}	57.59 ± 1.92^{a}	54.76 ± 2.51^{a}	40.29 ± 6.23^{b}
	T1	60.02 ± 2.50^{a}	59.06 ± 1.70^{a}	57.29 ± 2.26^{a}	53.89 ± 2.60^{a}	40.55 ± 6.33^{b}

Values are presented as mean ± standard deviation (SD). Different superscript letters (a–c) within the same row indicate significant differences among storage times (0–4 h) within each breed and treatment ($P < 0.05$) according to Duncan's multiple range test (DMRT). T0 = thawing at 37 °C for 30 s; T1 = thawing at 28 °C for 45 s; HF = Holstein-Friesian; Ongole.

Accordingly, evaluating post-thaw sperm quality under realistic field conditions is essential to improving AI efficiency. Previous studies have evaluated the effects of thawing methods and post-thaw storage at cold-to-moderate temperatures (approximately 4–17 °C) on sperm quality and fertility (Aksu *et al.*, 2024; García *et al.*, 2026; Nisa *et al.*, 2022). In general, frozen–thawed semen retains its fertilizing capacity only for a limited period (Botta *et al.*, 2019). However, prolonged storage has been associated with reduced motility, viability, and fertility, increased apoptosis, and decreased membrane and acrosome integrity (Koch *et al.*, 2022; Pozo-Curo *et al.*, 2026; Solís *et al.*, 2024). Unlike most previous work, the present study evaluated post-thaw storage at 37 °C and 28 °C, representing a physiological and more practical understanding of semen quality dynamics under routine AI conditions.

Overall, in this study showed that post-thaw storage duration was the primary factor affecting sperm quality, whereas the thawing method exerted only a relatively minor effect. Most parameters, including motility, viability, PMI, IAC, and TMS, declined with increasing storage duration. Thawing is a critical stage in the cryopreservation process that influences the structural stability of the sperm membrane (Athurupana *et al.*, 2015). During thawing, a suboptimal warming rate increases the risk of ice recrystallization, which may cause mechanical damage to the cell membrane. The use of a higher thawing temperature for a shorter duration can accelerate the warming rate, reduce ice recrystallization, and maintain membrane integrity (Nguyen *et al.*, 2023).

In contrast, lower temperatures for longer durations may reduce motility and increase acrosomal damage (Doležalová *et al.*, 2017). In this study, both thawing protocols (37 °C for 30 s and 28 °C for 45 s) yielded comparable results for all sperm quality parameters. Statistical analysis showed that the thawing method did not significantly affect most functional parameters, indicating that both protocols fall within a physiologically tolerable range. Consistent with these results, Fitriana *et al.* (2025) reported post-thaw motility above 40% under similar thawing conditions. Overall, the lack of significant differences between thawing treatments supports previous findings that moderate variations in thawing conditions do not adversely affect sperm quality (Zenteno *et al.*, 2023).

Marked differences among breeds were observed in post-thaw sperm performance, particularly in TMS and motility, indicating that sperm tolerance to cryopreservation and subsequent storage may vary among cattle genotypes. This finding is consistent with Wijaya *et al.* (2025), who reported that breed differences influence semen quality in Bali, Brahman, Ongole, Limousin, and Simmental cattle. Berry *et al.* (2019) reporting that variation in sperm motility is partly attributable to genetic heritability among cattle breeds. Such variation may reflect differences in genetic background, seminal plasma composition, and plasma membrane characteristics, which collectively influence sperm resilience during post-thaw storage.

The progressive decline in sperm motility and TMS with increasing storage duration likely reflects the availability of endogenous energy reserves in spermatozoa. Sperm movement requires a continuous supply of adenosine triphosphate (ATP), generated through mitochondrial

oxidative phosphorylation and glycolysis (Pramudhita *et al*., 2026). Prolonged post-thaw storage may impair mitochondrial function and cellular metabolism, thereby reduce ATP production and increase lactic acid accumulation. These metabolic alterations may impair flagellar activity and consequently reduce sperm motility (Abruzzese *et al*., 2024; Moraes *et al*., 2021). In addition, storage temperature and duration may influence the rate of motility decline by disrupting ionic homeostasis and cellular metabolism (Aksu *et al*., 2024; Botta *et al*., 2019). Pardede *et al*. (2020) reported that sperm motility of ≥ 40% is generally required for semen intended for artificial insemination.

In this study, PMI and viability declined progressively, indicating that sperm plasma membranes are highly sensitive to osmotic and oxidative stress during post-thaw storage. An intact membrane is essential for ionic balance and fertilization capacity (Suo *et al*., 2024; Varela *et al*., 2020). In contrast, membrane damage impairs nutrient transport, disrupts metabolism, and ultimately causes sperm death (Tethool *et al*., 2022). Although rapid thawing may lessen ice recrystallization damage, inappropriate temperatures or prolonged exposure can still induce membrane injury (Zenteno *et al*., 2023). Collectively, these results highlight the importance of preserving PMI to maintain sperm function.

In addition, the proportion of sperm with an IAC decreased with increasing storage time, indicating a progressive decline in fertilizing potential. The acrosome contains hydrolytic enzymes required for sperm penetration through the zona pellucida, therefore preservation of acrosomal integrity is essential for successful fertilization (Bernecic *et al*., 2021; Pérez Aguirreburualde *et al*., 2012). Prolonged storage may destabilize the sperm plasma and acrosomal membranes, promoting capacitation-like changes before the sperm reach the female reproductive tract. Such premature capacitation, often described as cryo-capacitation in frozen–thawed sperm, may predispose sperm to undergo a premature acrosome reaction, resulting in the early release of acrosomal contents, thereby reducing fertilization capacity (Syah *et al*., 2026). The decline observed in this study aligns with previous reports that cryopreservation and post-thaw handling can induce acrosomal damage (Jakop *et al*., 2023).

In contrast, sperm abnormality remained low and relatively stable throughout storage, indicating that short-term post-thaw storage affects sperm function more than morphology. All abnormality values were far below the 20% threshold considered acceptable for AI (Yendraliza *et al*., 2026). Most of the abnormality was secondary and associated with handling and cryopreservation, rather than primary defects arising from spermatogenesis (Arora *et al*., 2024; Perry, 2021). These results align with previous reports indicating that low abnormality levels do not necessarily confer high fertility when functional parameters decline (Aldini *et al*., 2022).

Sperm concentration remained stable across storage times, indicating that it is primarily determined by initial semen production and processing rather than post-thaw handling. This is consistent with previous findings that concentration is influenced by genetic background, ejaculate characteristics, and semen processing techniques (Sevilla *et al*., 2026). All values met the minimum standard of 25 million spermatozoa per straw (Wahyudi et al., 2025), confirming that concentration is a relatively insensitive indicator of short-term post-thaw deterioration (Tethool *et al*., 2022).

In the present study, TMS declined progressively with increasing post-thaw storage time across all breeds under both thawing treatments, primarily due to reduced sperm motility. In contrast, sperm concentration remained relatively stable throughout storage. Although Ongole semen showed the highest initial TMS values, marked reductions were observed in all breeds during prolonged storage. By 4 h, all breed treatment combinations had fallen below the minimum standard for AI. This decline is biologically plausible, as prolonged post-thaw holding may exacerbate sperm damage, impair plasma membrane function, and reduce flagellar activity, thereby lowering progressive motility (Weldon *et al*., 2026; Zenteno *et al*., 2023). Previous studies also reported higher TMS values immediately after thawing under similar thawing conditions (Yekti *et al*., 2023). According to Wahyudi et al. (2025), frozen semen is considered acceptable for AI when TMS is at least 10 million/straw based on this threshold, most breed–treatment combinations remained acceptable only up to 3 h of storage. Therefore, post-thaw storage duration appeared to be the main factor determining TMS decline, whereas the effect of warm-water and tap-water thawing was comparatively small and breed-dependent. Based on this threshold, most breed-treatment combinations remained acceptable for only up to 3 h, indicating that storage duration was the primary determinant of TMS decline. In contrast, the effect of the thawing method was limited and breed-dependent.

Overall, breed-related differences were consistently observed across all sperm quality parameters. Limousin and Simmental semen generally showed better resistance to post-thaw deterioration, whereas Bali semen showed lower tolerance, particularly during prolonged storage, while Ongole and Madura semen showed intermediate responses. These differences may be related to variation in genetic background, seminal plasma composition, and sperm membrane characteristics among breeds (Berry *et*

al., 2019; Ntemka *et al*., 2016; Shan *et al*., 2020). Prolonged storage also emphasized these breed-specific differences more clearly than thawing treatment, suggesting that intrinsic sperm resilience had a greater influence than thawing conditions. These findings indicate that frozen-thawed semen should ideally be used within 2 h after thawing to maintain optimal sperm quality. Therefore, efficient semen handling and minimizing post-thaw storage time are important to optimize AI success under field conditions.

CONCLUSION

This study demonstrated that post-thaw storage time was the primary factor driving deterioration in sperm quality in frozen semen from different cattle breeds. In contrast, the influence of the thawing method was generally modest and breed-dependent. Motility, viability, TMS, PMI, and IAC decreased progressively as post-thaw storage duration increased. In contrast, sperm abnormality remained low and stable, and sperm concentration was unaffected. Although both warm-water and tap-water thawing were feasible, their effects were inconsistent across breeds. Among the evaluated breeds, Limousin semen generally exhibited greater post-thaw resilience, whereas Bali semen tended to deteriorate most rapidly in several functional parameters. Overall, the findings indicate that frozen-thawed semen should be used as soon as possible after thawing within 2 h to maintain acceptable quality for AI.

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NOVELTY STATEMENT

This study provides novel comparative evidence on the post-thaw storability of frozen bull semen subjected to two field-applicable thawing protocols, warm-water thawing at 37 °C for 30 s and tap-water thawing at 28 °C for 45 s, across six cattle breeds. Unlike previous studies that mainly focused on immediate post-thaw quality or a limited number of breeds, this study simultaneously evaluated motility, viability, abnormality, sperm concentration, TMS, PMI, and IAC during 0–4 h of post-thaw storage. The findings demonstrate that post-thaw storage duration has a greater influence on sperm quality than the thawing method and provide practical evidence for semen handling for AI under field conditions.

AUTHOR'S CONTRIBUTION

APAY: Conceptualization, supervision, methodology, funding acquisition, writing original draft. TS: Conceptualization, supervision, methodology, validation. DAM, RRA, LAR: Data curation, formal analysis, writing review and editing. HAS: Investigation, visualization, writing review and editing. ADP, PU: Investigation, resources, writing review and editing. MSM: Methodology, investigation, validation, writing review and editing. AA, DS: Methodology, data curation, formal analysis.

Ethical clearance

This study was approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Brawijaya (Approval No. 221/EC/KEPK/07/2025). All procedures involving animals were conducted in accordance with relevant institutional guidelines and regulations for animal care and use.

Generative AI and AI-assisted technology statement

During the preparation of this manuscript, the authors used ChatGPT (OpenAI) to improve the quality of the written language. After using this tool, the authors reviewed and edited the manuscript as needed and remain fully responsible for the content of the published article.

Conflict of interest

The authors have declared no conflict of interest.

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