Research Article

Practical Use of Amino Acids in Diluent to Sustain Native Chicken Sperm Quality During 5 °C Storage

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Abstract | Cold storage of rooster semen is essential to support artificial insemination programs in native chickens; however, prolonged storage at low temperatures often induces oxidative stress that compromises sperm quality. This study aimed to evaluate the effects of glycine, glutamine, and histidine supplementation in semen diluents on the quality and longevity of native Indonesian chicken sperm stored at 5°C. Semen was collected from six mature native roosters (±10 months old) and diluted using a Ringer's lactate–egg yolk extender supplemented with glycine, glutamine, or histidine at concentrations of 20, 40, and 60 mM, with an unsupplemented diluent serving as the control. Sperm quality parameters, including motility, viability, plasma membrane integrity, acrosome integrity, DNA damage, and longevity, were evaluated after 24 and 48 hours of storage. The results showed that glycine, glutamine at various concentrations and histidine at a concentration of 20 mM did not cause differences in motility (35-42% at 24 hours of storage and 21-30% at 48 hours of storage) and sperm viability (84.12-89.17% at 24 hours of storage and 73.03-83.19% at 48 hours of storage). In contrast, histidine supplementation at 40–60 mM significantly reduced sperm motility, viability, and longevity. Plasma membrane integrity and acrosome integrity remained relatively stable across treatments, indicating lower sensitivity of these parameters to amino acid supplementation. In conclusion, glycine and glutamine are suitable additives for preserving native chicken semen quality during storage at 5°C, whereas histidine at high concentrations is detrimental. These findings provide practical guidance for optimizing liquid semen extenders in native chicken breeding programs.

Keywords | Amino acids, Cold storage, Native chicken, Semen quality, Sperm longevity

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INTRODUCTION

Indonesia possesses a high genetic diversity of native chickens; however, their productivity remains relatively low, resulting in continued dependence on imported broiler and layer chickens to meet national demand for meat and eggs. Improving the productivity of native chickens requires advances in nutrition, management practices,

and breeding programs (Hidayat and Asmarasari, 2015). Selective breeding is a key strategy to enhance growth performance, survival, fertility, and hatchability, and its effectiveness is strengthened by artificial insemination, which enables directed and efficient mating (Sapkota *et al*., 2020). Artificial insemination in poultry offers several advantages, including increasing the male-to-female mating ratio, allowing the use of older or injured roosters, preventing preferential mating, and supporting crossbreeding programs (Kharayat *et al*., 2016). In practical application, cold semen storage is essential for extending the usable duration of ejaculates. Storage at 5°C has been shown to better preserve mitochondrial activity and extend semen shelf life compared to moderate temperatures (Blank *et al*., 2021). Nonetheless, chicken spermatozoa are highly susceptible to cold-induced damage.

This susceptibility is closely associated with the high content of polyunsaturated fatty acids (PUFAs) in the plasma membrane, which increases vulnerability to oxidative stress and lipid peroxidation (Authaida *et al*., 2023; Barbarestani *et al*., 2024). Such oxidative damage compromises membrane integrity, disrupts the acrosome reaction, and ultimately reduces fertilizing capacity. A progressive decline in motility during 24-hour storage at 5°C has also been reported in native chicken semen (Khaeruddin *et al*., 2024a, c). Therefore, semen diluents require protective components, such as amino acids, which possess antioxidant properties and can reduce intracellular reactive oxygen species (Sangeeta *et al*., 2015; Davoodian *et al*., 2017; Koohestanidehaghi *et al*., 2021).

Amino acids including glutamine, alanine, serine, valine, and glycine play key roles in redox regulation and oxidative detoxification (Ugur *et al*., 2020) and are present in high concentrations in the seminal plasma of chickens (Santiago-Moreno *et al*., 2019). Previous studies have demonstrated that amino acids can preserve semen quality in mammals (Tan *et al*., 2024; Said *et al*., 2019) and poultry, including the effectiveness of glutamine in cryopreserved rooster semen (Khiabani *et al*., 2017) and glycine in chilled local chicken semen stored at 5°C for up to 120 hours (Junaedi *et al*., 2024). In human sperm, histidine consistently supports sperm motility over a long period (Hungerford *et al*., 2025).

To date, however, no study has directly compared the effects of glycine, glutamine, and histidine in semen diluents for native chickens stored at 5 °C. The novelty of this study lies in the simultaneous evaluation of these three amino acids at different concentrations to determine their distinct effects on motility, viability, membrane integrity, acrosome integrity, DNA stability, and the overall shelf life of chilled semen.

MATERIALS AND METHODS

Chicken maintenance and semen collection

Six mature Indonesian native roosters aged approximately 10 months were used in this study. The birds were individually housed in cages measuring 50 × 50 × 65 cm and were fed a complete ration containing 20% crude protein at 100 g/bird/day, with drinking water provided ad libitum. Semen collection was performed using the massage method as described by Kucera and Heidinger (2018), which involves massaging the tail region until the tail elevates and the papilla protrudes, followed by ejaculation. The semen was collected into a microtube using a small funnel. Ejaculate from six roosters was pooled in one tube and then divided into 10 tubes based on treatment.

Fresh semen evaluation

Fresh semen was evaluated macroscopically for volume, color, pH, and consistency. The pH was measured using a universal pH indicator strip (MQuant, Merck KGaA, Germany). Microscopic evaluation included sperm concentration, mass movement, motility, viability, and abnormalities. Sperm concentration was determined by diluting semen in 3% NaCl at a 1:500 ratio, placing the diluted sample on a Neubauer hemocytometer (Assistant, Germany), and observing it under a light microscope (Olympus CX33, Japan) at 100× magnification. Sperm abnormalities were assessed using eosin–nigrosin staining and examined at 400× magnification.

Diluent preparation

The diluent was prepared by mixing 90% Ringer's lactate solution (Widatra Bakti, Indonesia) with 10% egg yolk, followed by centrifugation at 2000 rpm for 30 minutes (Khaeruddin *et al*., 2024b). The supernatant was collected as the base diluent. Penicillin (1000 IU; Meiji, Indonesia) and streptomycin (1 mg/mL; Meiji, Indonesia) were added, and the pH was adjusted to match semen pH using tris-hydroxymethyl aminomethane. The diluent was divided into 10 tubes, and amino acids were added according to treatment. The treatments consisted of no amino acid (control) and the addition of glycine, glutamine, or histidine (Merck, Darmstadt, Germany) at concentrations of 20, 40, or 60 mM.

Dilution and storage

Semen meeting the quality requirement (motility > 70%) was divided into 10 tubes and diluted at a ratio of 1:5. The diluted semen was then stored at 5°C until all spermatozoa were dead (for longevity evaluation). Semen quality parameters were evaluated after 24 and 48 hours of storage.

Liquid semen evaluation

Liquid semen quality was assessed based on motility,

viability, plasma membrane integrity, acrosome integrity, DNA damage, and longevity. Sperm motility was evaluated subjectively under a light microscope at 400× magnification. Viability was assessed using eosin–nigrosin staining (Agarwal *et al*., 2016) on at least 200 spermatozoa.

Plasma membrane integrity was evaluated using the hypoosmotic swelling test (HOST). In this test, 10 µL of semen was mixed with 100 µl of HOST solution (0.9 g fructose and 0.49 g sodium citrate dissolved in 100 mL distilled water), incubated at 37°C for 30 minutes, and smeared on a slide using eosin–nigrosin. A minimum of 200 spermatozoa were observed at 400× magnification, with membrane-intact sperm identified by tail bending (Najafi *et al*., 2019; Khaeruddin *et al*., 2024c).

Acrosome integrity was examined using Coomassie Brilliant Blue (CBB) staining. Liquid semen was smeared on a slide, fixed in 5% formalin, air-dried, incubated at 37°C for 30 minutes, washed, air-dried, stained in 0.25% CBB (R250; BBI Life Sciences, Canada) dissolved in 10% glacial acetic acid and 25% methanol for 5 minutes, rinsed, and observed under a microscope at 1000× magnification with oil immersion (Silyukova *et al*., 2022; Khaeruddin *et al*., 2024c).

DNA damage was assessed using Toluidine Blue staining following Rui *et al*. (2017). Semen smears were air-dried, fixed in 96% ethanol–acetone (1:1) at 4°C for 30 minutes, air-dried for 30 minutes, hydrolyzed in 0.1 N HCl for 5 minutes at 4°C, rinsed three times with running water, air-dried, stained with Toluidine Blue for 20 minutes, rinsed, and dried. At least 200 spermatozoa were evaluated at 400× magnification; deep blue coloration indicated DNA damage, while light blue indicated intact DNA. Sperm longevity was recorded from the first day of storage until all spermatozoa lost viability.

Data analysis

The experiment used a completely randomized design (CRD) consisting of 10 treatments (types and concentrations of amino acids) with five replications (five separate semen collection days). Data were analyzed using Analysis of Variance (ANOVA) in SPSS version 25, followed by Duncan's Multiple Range Test when significant differences were detected.

RESULTS

Characteristics of fresh semen

Table 1 shows that the characteristics of fresh semen from native chickens fall within normal physiological ranges. The ejaculate volume (0.22 ± 0.04 mL), slightly alkaline pH (7.85 ± 0.22), milky appearance, and thick consistency indicate good semen quality. The high sperm concentration (5.58 ± 0.72 × 10^9/mL) and strong mass movement (++/+++) further reflect vigorous sperm activity, confirming that the samples were suitable for dilution and subsequent storage.

Table 1: Characteristics of fresh semen from native chickens.

Variable	Mean ± SEM
Volume (mL)	0.22±0.04
pH	7.85±0.22
Color	Milky
Consistency	Thick
Sperm concentration (10^9/mL)	5.58±0.72
Mass movement	++/+++
Motility (%)	80.00±2.89
Viability (%)	98.07±0.25
Abnormality (%)	3.65±0.36

Microscopic evaluations also demonstrated excellent semen quality, as indicated by high motility (80.00 ± 2.89%), very high viability (98.07 ± 0.25%), and a low abnormality rate (3.65 ± 0.36%). Overall, these values confirm that the initial semen used in this study was of optimal quality, ensuring that any changes observed during cold storage can be attributed primarily to the effects of the amino acid treatments rather than to poor initial semen condition.

Motility

Figure 1 shows that glycine and glutamine effectively maintained sperm motility during 24- and 48-hour storage at 5°C. At 24 hours, glycine at 40 mM and all glutamine concentrations produced the highest motility values, significantly outperforming the control. In contrast, histidine at 60 mM caused a marked decline in motility, indicating a detrimental effect at high concentrations.

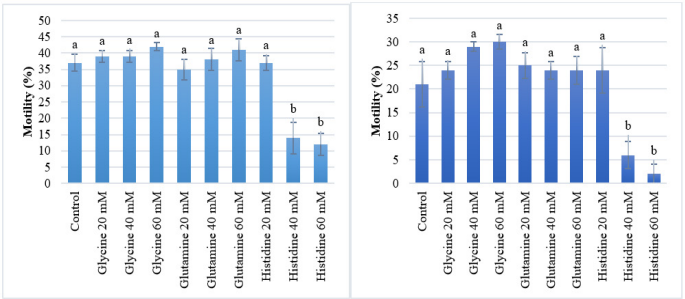

Figure 1: Sperm motility with the addition of various amino acids to the diluent for 24 hours (left) and 48 hours (right). Note: Different letters above the graph reflect significant differences (P<0.05).

After 48 hours, motility decreased across all treatments due to extended storage, yet glycine at 40 mM continued

to provide the strongest protective effect. Glutamine also maintained higher motility than the control, whereas histidine at 60 mM again resulted in the lowest motility. Overall, glycine particularly at 40 mM was the most effective additive, while high-dose histidine negatively affected sperm performance.

Viability

Figure 2 shows that both glycine and glutamine help protect rooster sperm from the stress of cold storage, allowing the cells to remain highly viable even after 24 and 48 hours at 5°C. Their presence in the diluent appears to support the sperm's resilience, keeping survival rates consistently high and comparable to fresh conditions. In contrast, histidine especially at 40 and 60 mM seems to overwhelm the cells, sharply reducing their ability to survive the cold. This contrast highlights how some amino acids nurture and preserve sperm vitality, while others, when given in excess, may instead compromise their stability.

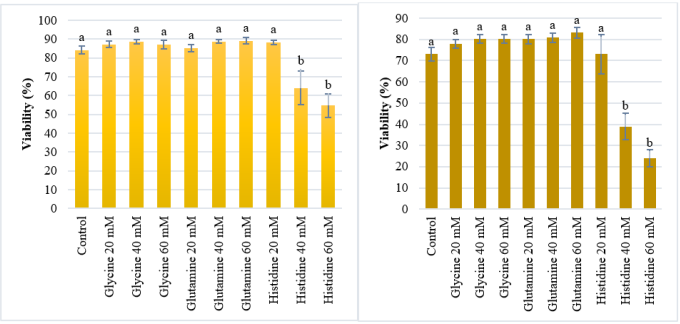

Figure 2: Sperm viability with the addition of various amino acids in diluent with 24 hours (left) and 48 hours (right) storage. Note: Different letters above the graph show significant differences (P<0.05).

Membrane integrity

Figure 3 shows that the plasma membrane integrity of rooster sperm remained consistently high after 24 and 48 hours of storage at 5°C. Most treatments including the control as well as glycine and glutamine at various concentrations maintained similar levels of membrane stability, indicating that the sperm cells were able to preserve their structural integrity during cold storage. Although histidine at higher concentrations produced a slight reduction in membrane integrity, the values were still within an acceptable range. Overall, these results highlight that the diluent, whether supplemented with amino acids or not, was effective in maintaining the fundamental integrity of sperm membranes throughout the storage period.

Acrosome intact

Figure 4 shows that sperm acrosome integrity remained well preserved during cold storage at 5°C, both after 24 and 48 hours, regardless of amino acid supplementation. All treatments, including the control, glycine, glutamine, and histidine at different concentrations, maintained similarly high percentages of intact acrosomes, with only a slight decline after 48 hours. These findings indicate that the acrosome is relatively resistant to cold-induced stress and that the addition of amino acids does not markedly influence acrosomal stability during short-term storage.

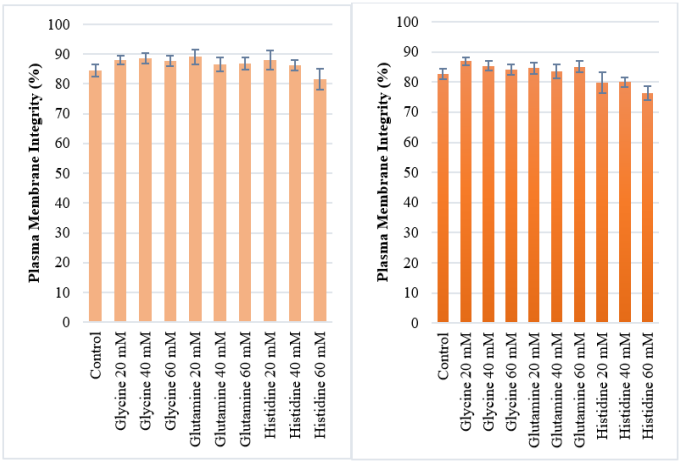

Figure 3: Sperm plasma membrane integrity with the addition of various amino acids in diluent, with 24 hours (left) and 48 hours (right) storage.

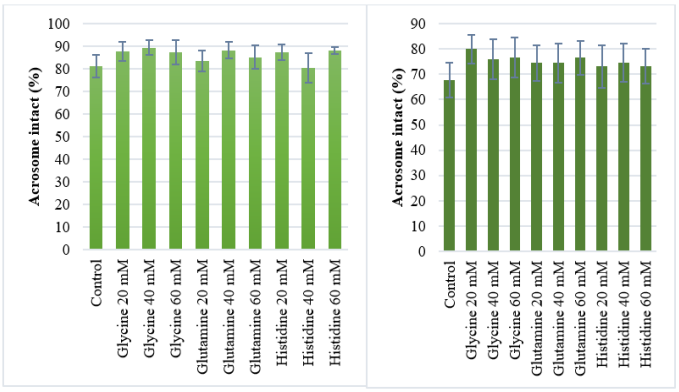

Figure 4: Intact sperm acrosome with the addition of various amino acids in diluents, with storage for 24 hours (left) and 48 hours (right).

DNA damage

Figure 5 shows that sperm DNA damage remained relatively low after 24 hours of storage and increased moderately after 48 hours at 5°C. At 24 hours, supplementation with glycine and glutamine tended to result in lower DNA damage compared with the control, indicating a protective effect on sperm genetic material. In contrast, histidine particularly at higher concentrations—showed a tendency toward increased DNA damage. After 48 hours of storage, DNA damage increased across all treatments as a consequence of prolonged cold exposure; however, glycine and glutamine continued to maintain lower levels of DNA damage than histidine. These results indicate that glycine and glutamine are more effective in preserving sperm DNA integrity during cold storage,

whereas high concentrations of histidine are less favorable for maintaining genetic stability.

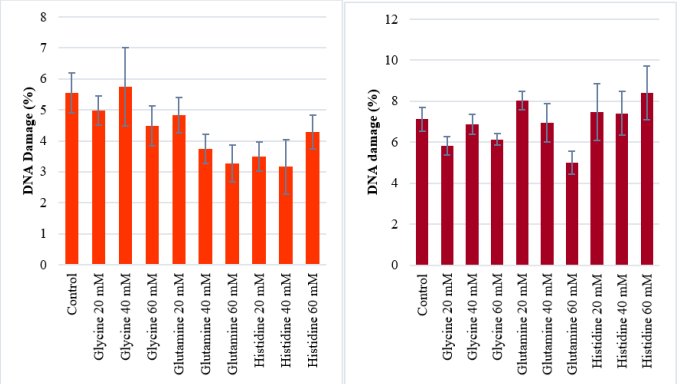

Figure 5: Sperm DNA damage with the addition of various amino acids in diluent with 24 hours (left) and 48 hours (right) storage.

Longevity

Figure 6 shows that glycine consistently maintained sperm longevity during storage at 5°C, with survival times comparable to or slightly longer than the control across all tested concentrations. Glutamine provided moderate support, allowing sperm to remain viable for a shorter period, while histidine markedly reduced sperm longevity, particularly at 40 and 60 mM. These findings highlight glycine as the most favorable amino acid for sustaining sperm survival during cold storage, whereas high concentrations of histidine compromise the ability of sperm cells to survive over time.

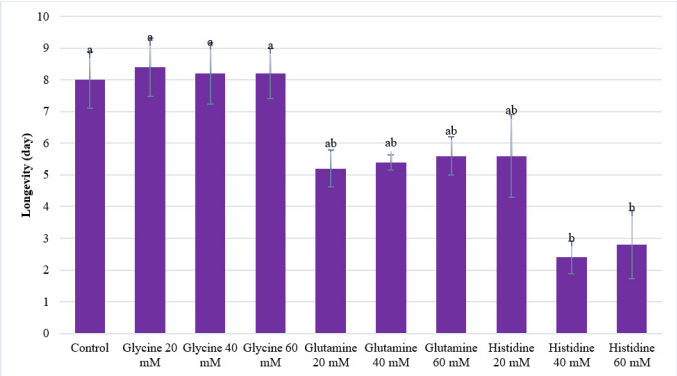

Figure 6: Sperm longevity at 5°C with the addition of various amino acids in diluent. Note: Different letters above the graph reflect significant differences (P<0.05).

DISCUSSION

This study aims to investigate the effect of adding glycine, glutamine, and histidine at multiple concentrations (20 mM, 40 mM, and 60 mM), to diluents on motility, viability, plasma membrane integrity, intact acrosome, DNA damage, and longevity of native Indonesian chicken sperm stored for 24 hours and 48 hours at 5°C. In this study, glycine did not show any difference in sperm quality compared to glutamine. These results correspond with the study on Sumba-Ongole cattle by Said *et al.* (2019), who reported that at the same concentration, glycine and glutamine did not produce any difference in sperm motility after cooling.

The addition of 40-60 mM histidine had no beneficial effect in this study, leading to lower sperm motility and viability. The result is consistent with the report by Matás *et al.* (2008), where the addition of L-histidine to diluent caused a significant decrease in boar sperm motility after 120 hours of storage. Furthermore, histidine was shown to have a negative effect on viability in goldfish sperm (Lahnsteiner, 2009).

The mechanism by which histidine caused the decrease in motility remains unclear, as no previous reports have described its negative effects on sperm. However, this may explain the effects in other cell types, such as in bacterial cells, where the addition of L-histidine increases the formation of intracellular hydroxyl radicals in *Escherichia coli* (Nagao *et al.*, 2018). Based on observation, excessive hydroxyl radicals can cause cell damage. Rauen *et al.* (2007) reported that hepatocyte cell culture media containing high concentrations of L-histidine caused hepatocyte cell inactivation in 3 hours with the occurrence of lipid peroxidation. In mammalian cells such as Chinese hamster ovary, L-histidine significantly increases H_2O_2 toxicity, causing more DNA double-strand breaks and severe mitochondrial damage. This implies that excess histidine worsens the effects of free radicals already present (Guidaerlli, 1995).

Based on observation, sperm quality declined during 48 hours of storage. Blank *et al.* (2021) reported reduced motility associated with decreased mitochondrial activity. Prolonged storage increased acrosome reaction values through elevated amidase activity and raised ROS levels, leading to lipid peroxidation (Ratchamak *et al.*, 2025), oxidative stress, mitochondrial membrane potential loss, impaired ATP production, and reduced motility (Słowińska *et al.*, 2018). In this study, glycine treatment led to motility of 42% after 24 hours and 30% after 48 hours, lower than values reported by Junaedi *et al.* (2024) for KUB chicken sperm (67.5% and 60% with egg yolk lactate Ringer diluent containing 60 mM glycine). Motility at 24 hours was also below reports for Thai native chicken sperm (81.96% with IGGKPh containing serine and 65.1% with BPSE containing vitamin B12) and at 48 hours below the 61.9% reported by Kheawkanha *et al.* (2023) and Suwimonteerabutr *et al.* (2024), respectively. Viability after 24 hours with glycine and glutamine treatments approached the 85.98% reported by Kheawkanha *et al.* (2023) in Thai native chicken sperm with IGGKPh containing serine. It

was higher than values for Lohman Brown sperm (76.59% with EM diluent) (Tvrdá *et al*., 2023), Thai native sperm (70.1% with BPSE) (Suwimonteerabutr *et al*., 2024), and Ross 308 sperm (60.5% with Lake diluent) (Alipour-Jenaghard *et al*., 2023). After 48 hours, viability with glycine and glutamine treatments remained higher than previous reports, at 67.9% (Suwimonteerabutr *et al*., 2024) and 30 (Alipour-Jenaghard *et al*., 2023).

Plasma membrane integrity in this research was higher than previously reported, with 61.5% after 24 hours of storage and 31.5% after 48 hours in Ross 308 Breeder chicken sperm using Lake diluent (Alipour-Jenaghard *et al*., 2023). In comparison, Suwimonteerabutr *et al*. (2024) reported 62.9% after 24 hours and 59.9% after 48 hours in Thai native chicken sperm using BPSE diluent. The proportion of intact acrosomes at 24 hours of storage was similar to the results of Tvrdá *et al*. (2023), who reported 81.91% in Lohman Brown chicken sperm using EM diluent, but lower than the values reported by Suwimonteerabutr *et al*. (2024), which were 96.8% at 24 hours and 95.8% at 48 hours in Thai native chicken sperm using BPSE diluent. The percentage of sperm with intact DNA in this research was also considerably higher than that reported by Tvrdá *et al*. (2023) in Lohman Brown chicken sperm using EM diluent.

CONCLUSION

In conclusion, the addition of various types and concentrations of amino acids affects the motility, viability, and longevity of native Indonesian chicken sperm during storage at 5°C. The use of glycine and glutamine at all concentrations (20, 40, and 60 mM), as well as 20 mM histidine in the diluent did not show differences in sperm motility and viability compared to without amino acids. Meanwhile, approximately 40-60 mM histidine decreased sperm motility, viability, and longevity.

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NOVELTY STATEMENT

This study was the first to use a diluent containing glutamine and histidine in liquid-stored chicken semen. This study examined the effect of adding amino acids to the diluent on the longevity of chicken sperm stored at 5°C. The results showed that 40-60 mM histidine reduced chicken sperm quality during storage.

AUTHOR'S CONTRIBUTION

KK: Research design, conducting research, data analysis, writing the manuscript. HH: Literature search, conducting research. BS AHF and AK: Methodology and supervision AM, MEK, DY and RA: Editing and revising the manuscript. P: Assisting in conducting research.

Generative AI and AI-assisted technology statement

The authors declare that no generative AI or AI-assisted technologies were used in the preparation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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