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Exploring enzymatic antioxidant activities in Asian Elephant (*Elephas maximus*) sperm and seminal plasma

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ABSTRACT

This study explored the relationships between sperm quality and the activities of antioxidant enzymes (superoxide dismutase, SOD, and glutathione peroxidase, GPx) in sperm (S) and seminal plasma (SP) of Asian elephants, as well as the potential protective role of glutamine in semen extenders during chilled storage. In experiment 1, twenty ejaculates from 11 bulls were analyzed for percentages (%) of sperm motility (%MOT), viability (%VIA), and activities of SOD and GPx. A significant negative correlation was found between %VIA and SP-SOD ($r = -0.71$), and positive correlations between %MOT and both S-GPx ($r = 0.49$) and SP-GPx ($r = 0.57$). Following these findings, glutamine, a precursor of glutathione and semen antioxidant was introduced in Experiment 2. Each ejaculate (in a total of 8 ejaculates obtained from four bulls) was diluted in Tris-fructose-(EYT) and Tris-glucose-egg yolk extender (TCG), respectively, supplemented with 0 (control), 20 and 40 mM glutamine and stored at 4 °C. Sperm parameters including %MOT, %VIA, functional membrane integrity (%sHOST), and DNA integrity (%DNA) were assessed after 24 and 48 h of storage. Results demonstrated that glutamine did not significantly affect %VIA or %DNA. A significant reduction in %MOT was observed at 24 h, but 20 mM glutamine added in extenders reversed this decline at 48 h. A 40 mM addition of glutamine in TCG markedly reduced %sHOST at 24 h. In conclusion, SOD and GPx activities in the sperm and seminal plasma are indicative of sperm quality. During chilling, using a higher level of glutamine may damage the spermatozoa of Asian elephants.

1. Introduction

Asian elephants are considered endangered according to the International Union for Conservation of Nature (IUCN), with less than one-third of the global population being in captivity [1]. In Thailand, the number of captive-managed elephants has witnessed a drastic 95 % reduction over the last century [2]. Currently, captive and wild populations in this region have stabilized roughly equal numbers, with around 4000 individuals in each group. However, a concerning trend today has emerged that captive populations are not self-sustainable in elephant-ranging countries [3]. A population viability analysis (PVA) has revealed a significant decline in the *ex-situ* birth rate in Thailand. This disparity poses a threat to elephant conservation, and should mortality rates increase by 6 %, this species could become extinct within 420 years [4]. Intensive research and development of *ex-situ* breeding programs are imperative to address the limitations of *in-situ* conservation and ensure the long-term survival of this endangered species.

Adopting robust conservation strategies and effective breeding management of captive Asian elephants is critical to improving reproductive success [5]. A significant challenge within captive populations is the selection of suitable males for breeding, complicated by variability in sperm quality, even within the same individual [6,7]. The reasons for

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this disappointing semen quality are poorly understood, presenting a gap in the current scientific knowledge [7,8]. To address these reproductive challenges, the storage of semen in chilled or frozen conditions has been adopted in elephants [9–11]. This approach mitigates issues of substandard semen quality in certain areas and allows for the distribution of genetic materials over vast distances among elephant herds, enhancing genetic diversity. Furthermore, the use of artificial insemination (AI) has been increasingly incorporated into breeding programs to improve fertility [12–14]. In other species, the use of semen extenders has demonstrated effectiveness, improving cryopreservation [15,16], and positively influencing AI outcomes [17], *in vitro* fertilization results [18] and pregnancy rates [19]. However, the sperm of Asian elephants are particularly sensitive to temperature fluctuations during semen preservation [6,7,20,21], which can adversely affect motility, viability, DNA integrity and acrosomal integrity [7,22]. Currently, there is no semen extender that effectively maintains the quality of elephant sperm during chilling.

Mammalian spermatozoa, abundant in polyunsaturated fatty acids (PUFAs), are highly susceptible to oxidative stress from reactive oxygen species (ROS) during chilled storage [23]. An imbalance between ROS and antioxidant defenses can lead to significant lipid peroxidation [24], thereby compromising sperm quality. ROS detected in sperm cells include superoxide radical (O^{2-}), hydroxyl radical (OH^-), oxygen (O_2) and hydrogen peroxide (H_2O_2) [24]. To mitigate the detrimental effects of these ROS, enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) are essential in counteracting oxidative stress within spermatozoa and seminal plasma [25–28]. Specifically, SOD can transform the O^{2-} into H_2O_2, and GPx can transform H_2O_2 and other hydroperoxides created by SOD scavenging system into H_2O, thus preventing oxidative damage to the spermatozoa [29]. The efficacy of these antioxidant systems is vital for maintaining sperm quality during chilled storage [26]. Without adequate antioxidants to counterbalance this process, lipid peroxidation can detrimentally affect sperm quality, as evidenced across various species including Asian elephants [20], boars [30], buffaloes [31] and canines [32]. Incorporating antioxidants into semen extenders can reduce ROS stress during chilling and freezing processes [33–35]. One such antioxidant, glutamine, is a key amino acid metabolized to glutamate [36]. Glutamate is then combined with cysteine and glycine to form glutathione (GSH), a tripeptide that serves as the substrate for GPx. Studies have shown that glutamine is effective in reducing ROS in sperm cells during cryopreservation and chilled liquid preservation [37,38]. However, the impact of glutamine varies among different species. For instance, studies have shown beneficial effects when glutamine is incorporated into semen extenders for buffalo [39], boar [40], rabbit [41] and rooster [42] before freezing. Conversely, elevated concentrations of glutamine have been observed to adversely affect sperm quality in bulls and stallions [43,44]. This variability underscores the necessity for species-specific studies to determine the optimal levels of glutamine required to preserve sperm quality effectively. Currently, there is a lack of research on the effectiveness of extenders containing antioxidants like glutamine in preserving the quality of elephant sperm during chilled storage.

The objectives of this study were to 1) determine the correlations between sperm motility and viability with antioxidative activities of SOD and GPx in the sperm cells and seminal plasma; 2) identify the optimal levels of glutamine in Tris-fructose- (EYT) and Tris-glucose-egg yolk extender (TCG) for maintaining Asian elephant sperm quality during storage at 4 °C for 48 h.

2. Materials and method

2.1. Animals, semen collection and evaluation

All animal use and procedures were approved by the Institution Animal Care and Use Committee, Faculty of Veterinary Science, Chulalongkorn University (No. 2031044).

In Experiment 1, a total of 20 ejaculates were collected from 11 elephant bulls, including 5 bulls housed at the National Elephant Institute (NEI), Forest Industry Organization, Lampang, Thailand, and 6 bulls housed at Maesa Elephant Camp and Maetaman Elephant Camp, Chiang Mai, respectively. The ages of the bulls ranged from 10 to 50 years, with a mean age of 34.5 ± 11 years. In Experiment 2, eight ejaculates were obtained from four bulls held at the NEI, ranging between 12 and 46 years of age (averaged 30.8 ± 17.3 years).

Ejaculates were obtained by performing the rectal massage as previously described [45]. All bulls were well-trained for semen collection. Throughout the semen collection process, the mahouts (elephant handlers) accompanied and controlled the elephants. An experienced leader of the mahout team supervised and monitored each sample collection to maintain consistency and accuracy. The rectal massage was performed until the elephants urinated, indicating the completion of the procedure. During collection, the tubes (more than 10) used to collect semen were frequently replaced. Ejaculates that exhibited visual or olfactory signs of urine contamination were discarded. After each collection session, the elephants were allowed to rest for a minimum of three days, with a weekly rest period incorporated. Each ejaculate was evaluated immediately for color and volume. Sperm concentration was determined by a hemocytometer chamber (Boeco, Hamburg, Germany). Semen pH was determined by a pH meter (HANNA Instruments, Woonsocket, Rhode Island, USA). All sperm evaluations were conducted by one expert and three theriogenologists to ensure accuracy and reliability.

2.1.1. Sperm motility and viability

To determine sperm motility, a 5-μL semen sample was dropped onto a glass slide preheated to 37 °C. The slide was covered with a coverslip, and the sperm were observed using a phase contrast microscope (Olympus, Shinjuku, Japan) at 200 × magnification. Sperm viability was assessed using eosin-nigrosin staining. In total of 200 sperm per sample were assessed. Only spermatozoa with no staining were classified as having an intact membrane (referred to as live spermatozoa) [46].

2.1.2. Plasma functional membrane integrity

The functional integrity of the sperm plasma membrane was determined using a short hypo-osmotic swelling test (sHOST) [47]. Briefly, 20 μL of semen was incubated at 37 °C in 200 μL of hypo-osmotic solution (75 mOsm/kg; 1:10, v/v) for 1 h. Following incubation, the semen was fixed using a hypo-osmotic solution containing 5 % (v/v) formaldehyde (fixed mixture) (Merck, Darmstadt, Germany). Subsequently, a slide was warmed to 37 °C, and a 5 μL drop of the fixed mixture was applied onto it, covered with a coverslip. Sperm (n = 200) per slide were evaluated under a phase-contrast microscope (400 ×). Spermatozoa displaying coiled tails were identified as maintaining intact functional membrane integrity, indicating a positive outcome in the sHOST.

2.1.3. DNA integrity

Sperm DNA integrity was assessed based on Acridine orange (AO) fluorescence, with modifications to the methods previously reported [48,49]. Briefly, a volume of 8–10 μL of diluted semen was spread onto a glass slide and allowed to air-dry on a slide warmer maintained at 37 °C. The dried slides were then fixed overnight at room temperature using Carnoy's solution, consisting of methanol and glacial acetic acid in a 3:1 (v/v) ratio. Subsequently, the prepared slides were air-dried again and stained with a 1 % (100 mg/mL) AO solution for 10 min. The AO staining solution was freshly prepared daily, combining 10 mL of 1 % AO with 40 mL of 0.1 M citric acid and 2.5 mL of 0.3 M $Na_2HPO_4{\cdot}7H_2O$, all from Merck, Darmstadt, Germany. The staining process was carried out in a dark environment at room temperature. After staining, the slides were gently rinsed in a stream of distilled water. Sperm (n = 200) per slide were examined using a fluorescent microscope (BX51, Olympus, Japan) at 1000 × magnification oil immersion. Spermatozoa displaying green fluorescence in the head were categorized as having intact DNA

(double-stranded), while those exhibiting red, yellow, orange, or mixed fluorescence were considered as having DNA damage [50].

2.2. Experiment 1: activities of antioxidant enzymes (SOD and GPx) in the sperm and seminal plasma

2.2.1. Seminal plasma and sperm cell collection

Semen samples were subjected to centrifugation at 3000 × *g* for 10 min to separate sperm cells from seminal plasma. The supernatant was again centrifuged at the same conditions until the sperm-free seminal samples were collected. The obtained sperm cells were washed twice with normal saline at 600 × *g* for 5 min each time to remove any residual components.

2.2.2. Determination of enzyme activity

Enzyme extraction was performed using a hypo-osmotic-thermal shock method [32]. The sperm pellet obtained was re-suspended with 1 mL of normal saline and centrifuged at 600 × *g* for 5 min, repeated twice. The supernatant was discarded and the sperm pellet was resuspended in double distilled water. A cold shock was applied by cryopreserving the sample at − 20 °C for 2 h, followed by thawing at room temperature. After thawing, the sample was centrifuged at 20,000 × *g* at 4 °C for 20 min. An aliquot (0.5 mL) of the supernatant was stored at − 20 °C until assays.

Superoxide dismutase (SOD) activity was measured by using a commercially available kit (RANDOX Laboratories, Crumlin, UK) following the manufacture protocol [27,51]. Superoxide radicals are generated by the xanthine-xanthine oxidase system react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. SOD activity was defined as the amount causing 50 % inhibition of INT reduction. Briefly, 0.05 mL of 10-fold diluted seminal and extracted antioxidative enzyme sample with Ransod sample diluents was thoroughly mixed with commercial test kit-mixed substrate (0.05 mM/L xanthine and 0.025 mM/L). After adding 0.25 mL of xanthine oxidase, SOD activity was measured spectrophotometrically at 505 nm.

Glutathione peroxidase (GPx) activity was manually measured by a Ransel Kit (Randox Laboratories Ltd, Crumlin, UK) [51]. GPx catalyzes glutathione (GSH)-cumene hydroperoxide reaction to oxidized GSH (GSSG). The reaction between GSSG, nicotinamide adenine dinucleotide phosphate (NADPH) reduction, and glutathione reductase (GR), NADP+ is generated. The GPX activity level was indirectly measured by the increase in NADP+ level. Briefly, a 0.02 mL of 10-fold diluted seminal and extracted antioxidative enzyme sample with Ransel sample diluents was thoroughly mixed with reagent I (4 mM/L GSH, 0.5 U/L GR and 0.34 nM/L NADPH) and 0.18 mM/L cumene hydroperoxide. The absorbance was recorded at 340 nm. The activities of SOD and GPx were expressed as U/L in the seminal plasma and U/10^6 in the spermatozoa, respectively.

2.2.3. Statistical analysis

Data were analyzed with Statistical Analysis System version 9.4 (SAS Institute, Cary, NC, USA). Spearman's correlation (*r*) was performed to detect correlations among sperm motility, sperm viability, GPx and SOD activities. The statistical significance was set at $P < 0.05$.

2.3. Experiment 2: the effects of glutamine with extenders on chilled sperm quality

2.3.1. Extenders

Two extenders were selected in the present study. Tris-glucose-egg yolk extender (TCG): composed of 2.4 g Tris, 1.4 g citric acid, 0.8 g glucose, 100,000 iu NA-benzyl penicillin, 0.1 g streptomycin sulfate, 20 mL egg yolk and added distilled water to 100 mL, pH 6.4 [52]. Tris-fructose-egg yolk extender (EYT): 3.025 g tris, 1.7 g citric acid, 1.25 g fructose, 0.06 g benzylpenicillin, 0.1 g streptomycin sulfate, 20 mL egg yolk and added distilled water to 100 mL, pH 6.78 [53].

2.3.2. Semen processing

Semen was centrifuged at 3000 × *g* for 10 min to separate the supernatant and seminal plasma. Ejaculates exhibiting initial motility ≥ 30 % were selected and diluted slowly with TCG and EYT to a final concentration of 100 × 10^6 spermatozoa/mL at room temperature. Subsequently, the diluted semen was divided into three separate aliquots, each treated with different concentrations of glutamine (0, 20, and 40 mM). Samples were incubated in the refrigerator (4 °C) and the sperm parameters (Sperm motility, %MOT; viability, %VIA; plasma functional membrane integrity, %HOSTs and DNA integrity, %DNA) were examined at three-time points: 0, 24, and 48 h.

2.3.3. Statistical analysis

All statistical analyses were performed using Statistical Analysis System version 9.4 (SAS Institute, Cary, NC, USA). The normality of distribution was measured by the Kolmogorov-Smirnov test. Descriptive statistics including several non-missing values, mean, standard deviation and range of the continuous data were calculated using the MEANS procedure of SAS. The effects of extenders (TCG and EYT), storage time (0, 24 and 48 h), and glutamine (0 [control], 20, and 40 mM) and their interactions on %MOT, %VIA, %sHOST and %DNA of elephant spermatozoa were determined by the Mixed Procedure of SAS. The statistical models included the fixed effects of extender, storage time, glutamine and their interactions, and a random effect of bull. Least square mean was obtained from the model and compared using least significant difference test. A statistically significant difference was defined as $P < 0.05$.

3. Results

3.1. Experiment 1

The mean ± SD of the percentages (%) motility (%MOT) and viability (%VIA) across 20 ejaculates from 11 bulls were 15.2 ± 3.9 % and 43.9 ± 4.7 %, respectively. The colors of the collected semen varied from opalescent to milky and creamy, with pH values ranging from 5.42 to 9.0 (mean ± SD, 7.4 ± 0.2). Sperm concentration ranged from 280 to 4755, with a mean of 1285.8 ± 246.4 × 10^6 spermatozoa/mL. The level (mean ± SEM) of GPx activity in the sperm and seminal plasma were 253.9 ± 1.9 U/10^6 spermatozoa (range: 239.5–279.5) and 250.3 ± 1.4 U/L (range: 238.0–261.2), respectively. The level of SOD activity in the sperm and seminal plasma were 0.26 ± 0.06 U/10^6 spermatozoa (range: 0–0.96) and 24.4 ± 0.5 U/L (range: 24.2–27.5) in the sperm and seminal plasma, respectively.

There was a significant positive correlation between %MOT and GPx activity in both sperm (r = 0.49, P = 0.03) and seminal plasma (r = 0.58, P = 0.01; Table 1). Conversely, %VIA showed a significant negative correlation with SOD activity in seminal plasma (r = − 0.72, P < 0.01). No significant correlation was observed between the activities of GPx and SOD within either the sperm or seminal plasma (P > 0.05).

Table 1
Correlation coefficient (*r*) between sperm parameters, percentage of motility (%MOT) and percentage of viability (%VIA) and the enzyme activities in the sperm (U/10^6 spermatozoa s) and seminal plasma (U/L) of 11 Asian elephant bulls (n = 20 ejaculates).

Sperm parameters	GPx		SOD	
	Sperm	Seminal plasma	Sperm	Seminal plasma
%MOT	0.49*	0.58*	0.13	-0.26
%VIA	0.27	0.30	-0.12	-0.72*

Abbreviations: GPx, glutathione peroxidase; SOD, superoxide dismutase; %MOT, percentage of sperm motility; %VIA, percentage of sperm viability.
* Statistically significance at P < 0.05.

3.2. Experiment 2

The initial sperm characteristics of four bulls (n = 8 ejaculates) are summarized in Table 2. The results showed a significant effect of storage duration on sperm quality, with all measured parameters showing a marked decrease throughout the 48-h storage period at 4 °C ($P < 0.001$).

No significant differences were found in chilled sperm parameters (%MOT, %VIA, %sHOST, and %DNA) for various time points (at 0, 24 and 48 h) between the TCG and EYT extenders ($P > 0.05$). Furthermore, glutamine supplementation to both TCG and EYT did not influence %VIA or %DNA ($P > 0.05$). A considerable reduction in %MOT occurred at 24 h with glutamine addition in both extenders, dropping to nearly half. However, a concentration of 20 mM glutamine had a beneficial effect on %MOT after 48 h of storage compared to 24 h. Specifically, the addition of 20 mM glutamine in EYT significantly improved %MOT after 48 h ($P < 0.05$, Table 3). No significant difference in %MOT was observed between the control group and the 20-mM glutamine group. A significant decrease in %sHOST was noted with glutamine in both extenders at 48 h of storage. A marked reduction in %sHOST was seen at 24 h with the addition of glutamine to TCG ($P < 0.05$, Table 3).

4. Discussion

In recent years, there has been a decrease in the amount of research into why spermatozoa of Asian elephants respond undesirably to chilled liquid preservation. To the best of our knowledge, this study is pioneering in demonstrating correlations between the quality of sperm and the activities of two key antioxidant enzymes—superoxide dismutase (SOD) and glutathione peroxidase (GPx)—within the sperm (S) cells and the seminal plasma (SP). Furthermore, the negative impact of higher concentrations of glutamine supplementation on chilled spermatozoa of Asian elephants was demonstrated.

A significant positive correlation between SP-GPx activity and sperm motility was found, suggesting GPx is likely a key component in the enzymatic defense against motility degradation of Asian elephants. These findings are consistent with the previous studies in humans and buffaloes [54,55]. At physiological levels, ROS are essential for normal sperm function and facilitate sperm-oocyte binding, which is necessary for fertility [56], yet in excess, this abnormally high level causes oxidative stress which subsequently damages sperm cells. However, SP-SOD activity was negatively correlated with sperm viability in the present study. This contradicts the findings of the previous studies, which indicated that SP-SOD activities are positively associated with sperm viability and motility in buffaloes and humans [55,57]. Similarly, donkeys [26], dogs [58], and humans [59] may use higher SOD activity to prevent sperm motility loss. In addition, in humans, SOD rather than GPx helps to protect sperm from lipid peroxidation by neutralizing superoxide radicals [60]. However, in the current study, SOD did not appear to have a notable protective effect on the sperm of Asian elephants.

Interestingly, GPx and SOD activities in the sperm were detected. In addition, S-GPx was found to be positively correlated with sperm motility, suggesting elephant spermatozoa, similar to other species [27], may also use these systems to mitigate the detrimental effects of ROS. On the other hand, S-SOD did not show an impact on sperm quality and was present at much lower levels compared to its activity in seminal plasma. This could imply that SOD in the sperm cells might be overwhelmed by oxidative stress. Additionally, the composition and activity of antioxidant enzymes in sperm and seminal plasma are known to vary widely among different species [25,26,51,61,62], as well as within individuals of the same species [55,63]. Taken together, GPx appears to play a major role in the antioxidant system of elephant semen, highlighting the need for more comprehensive research to understand the distinct roles of GPx and SOD in elephant sperm function.

Table 2
Initial sperm characteristics of 4 Asian elephant (n = 8 ejaculates) prior to chilled in glutamine supplemented extender.

Sperm characteristics	Mean ± SD	Range
Concentration ($\times 10^6$ spermatozoa /mL)	1003.3 ± 526.3	1035.0–1888.0
pH	7.0 ± 0.7	6.0–8.8
Volume (mL)	33.5 ± 15.7	8.0–55.1
Motility (%)	31.9 ± 23.7	30.0–55.0
Viability (%)	44.4 ± 19.5	11.0–69.0
Intact functional membrane integrity (%)	23.1 ± 10.5	2.5–37.5
Normal DNA integrity (%)	58.3 ± 19.0	23.0–88.0

No differences were found between the use of TCG and EYT. A previous study has documented that egg yolk-based extenders can effectively preserve sperm viability in Asian elephants during a 48-hour chilling period, particularly when initial sperm motility exceeds 70 % [64]. However, in this study, the criterion for initial motility was lowered to less than 30 %, with an average of 31.7 ± 23.7 %. Typically, the ideal initial motility for chilled semen ranges between 50 % and 80 % in Asian elephants [11,20], buffaloes [65], and rams [66]. Achieving sperm motility above 70 % in elephants is particularly challenging [7], with reported starting motility often under 11.7 % [6] and 30 % [22], respectively. Several factors contribute to the low percentage of motile spermatozoa, including seasonal variations (particularly during summer) and old age [67], and infrequent rectal stimulations [68], *etc.*, contribute to a low percentage of motile spermatozoa. The underlying factors contributing to poor semen quality in elephants are not fully understood.

The compromised initial motility may probably be held accountable for a marked deterioration in all sperm parameters observed in this study. Asian elephant spermatozoa are particularly sensitive and vulnerable to oxidative stress, probably due to the high abundance of docosahexaenoic acid [69]. This decline could also be linked to ROS-induced damage caused by lipid peroxidation during chilled storage [23], which can harm sperm quality in humans [70] and Asian elephants [20]. However, this study did not investigate relationships between oxidative damage and chilled storage sperm of elephants. In addition to the poor initial sperm motility, the formulation of an extender is likely to play a key role in improving the higher survival rates of spermatozoa. TCG and EYT were chosen for this study due to the practicality of field preparation. TCG has been previously employed for diluting elephant semen [10,20], while the EYT is commonly used for the chilled storage of canine semen [53]. Other commercial semen extenders for elephants have been shown to enhance sperm resilience during liquid preservation across various temperatures [7,22,71]. A key difference between the two tested extenders is the type of sugar used glucose or fructose. Research has indicated that trehalose can have a beneficial effect on sperm motility compared to monosaccharides [72]. Moreover, it has been observed that monosaccharides but not glucose, can improve acrosome integrity and motile rate of spermatozoa [73]. Although proper semen treatment during chilled storage is speculated to aid in the success of freezing [11,30,71], no extenders have yet been proven to be highly effective for elephant semen preservation.

Introducing glutamine into the TCG at a concentration of 40 mM negatively impacted sperm membrane integrity when stored at 4 °C for 24 h, possibly suggesting an adverse interaction with egg yolk components within the extender. Different concentrations of egg yolk seem to contribute to various responses [74,75]. The optimal glutamine concentration for maintaining sperm quality during cryopreservation [43,44] and liquid preservation [38,76] is still debated. The present findings are similar to Thai native horses, high levels of glutamine decreased sperm motility and membrane integrity during chilling [77], possibly due to the toxic effect of inducing osmotic dehydration and subsequent cell injury [44,78]. In stallion semen, supplementation with 50 mM glutamine during freezing was associated with decreased sperm quality [79]. A higher concentration, from 80 to 100 mM, proved toxic to frozen-thawed sperm quality in bulls [43], cynomolgus monkeys [80] and stallions [44].

Table 3
Comparisons of elephant sperm characteristics chilled in two types of extenders supplemented with various concentrations of glutamine for 48 h (n = 4 bulls, 8 ejaculates).

Time (h)	Glutamine (mM)	TCG				EYT			
		%MOT	%VIA	%sHOST	%DNA	%MOT	%VIA	%sHOST	%DNA
0	0	33.07[a]	44	23.4[a]	57.3	35.6[a]	43.6	22.4[a]	51.2
	20	33.1[a]	43.3	20.1[a]	55.1	35.6[a]	43.7	20.8[a]	51.3
	40	31.2[a]	43.7	19[a]	54	330.1[a]	45.1	20.2[a]	49.4
SEM		9.7	11.8	3.1	9.7	9.7	11.8	3.1	9.7
24	0	17.6	36.3	12	47.4	17.5	35.7	13.8	48
	20	16.2	36.4	9.2	49.4	18.1	36.5	10	48.7
	40	13.3	35.3	8.3[c]	46.8	14.3	36.7	8	46.6
SEM		9.7	11.8	3.1	9.7	9.7	11.8	3.1	9.7
48	0	21.5[a]	43.4	11.1	47	21.5[a]	45	11.3	49.6
	20	21.5[a]	42.7	4.9	48.7	22.7[a]	46.6	6.1	50.1
	40	15.2	42.6	6.6	46.3	14[a]	42.2	4.6	45.7
SEM		11	12.8	3.9	10.8	11	12.8	3.9	10.8
P-value		*	-	*	-	*	-	*	*

Abbreviations: %MOT, percentage of sperm motility; %VIA, percentage of sperm viability; %sHOST, percentage of intact functional sperm membrane integrity; %DNA, percentage of sperm DNA integrity; TCG, Tris-glucose-egg yolk extender; EYT, Tris-fructose-egg yolk extender; standard error of mean, SEM.
Data were presented as Least Square Means.
[a] Different superscript letters in the same column are different (*$P < 0.05$).

The findings of this study contrast with earlier research which suggested that 20 mM of glutamine in boar [38] and ram [81] semen could preserve chilled sperm. Apart from its predominant presence in testicular fluid [82], glutamine also is the most prevalent amino acid in the seminal plasma of bulls and chickens, though the levels vary [83,84]. In other species, lower concentrations of glutamine supplementation have demonstrated different outcomes. For instance, compared to higher concentrations, rooster semen preserved with 2.5 mM glutamine displayed better frozen-thawed membrane integrity and normal morphology of sperm [42]. In rabbits, a concentration range of 0.5–2.0 mM of glutamine was employed, with 2 mM showing enhanced sperm acrosome integrity [41]. The discrepancies observed may be attributed to the distinct secretions, composition, and volume from accessory glands across species, which affect seminal plasma composition and, consequently, sperm's response to glutamine supplementation, as observed in goats [85] and humans [86]. Given these findings, the present research prompts further investigation into whether lower glutamine concentrations might preserve elephant spermatozoa under a chilled condition.

In conclusion, this study has confirmed, for the first time, that both sperm and seminal plasma from Asian elephants exhibit activities of GPx and SOD, which are linked to sperm quality. GPx in elephant semen, in particular, seems to be integral to the enzymatic defense against oxidative stress. However, the precise levels of these enzymes that are optimal for sperm health were not determined within the scope of this research. Additionally, the study found that adding glutamine to TCG and EYT extenders does not protect elephant sperm during short-term chilled storage. Higher levels of glutamine were detrimental to the integrity of the sperm plasma membrane.

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CRediT authorship contribution statement

Padet Tummaruk: Formal analysis. **Nuttawadee Ngamlertwong:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Patharapol Piamsomboon:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Nicole Sirisopit Mehl:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Taweepoke Angkawanish:** Supervision, Resources, Investigation. **Junpen Suwimonteerabutr:** Methodology, Investigation, Conceptualization. **Yuqing Yang:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Kaywalee Chatdarong:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Phakjira Sanguansook:** Methodology, Investigation. **Pakpoom Navanukraw:** Methodology, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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