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HOW DO TAURINE AND ERGOTHIONEINE ADDITIVES IMPROVE THE PARAMETERS OF HIGH- AND LOW-QUALITY TURKEY SEMEN DURING LIQUID PRESERVATION?*

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Abstract

The study aimed to examine the parameters of turkey ejaculates (n=40) of high and low quality (HQ and LQ, respectively), preserved with the addition of taurine (TAU; 5 mM, 10 mM) and ergothioneine (EGT; 5 mM, 10 mM) for 48 h at 5°C in a liquid state. The motility, plasma membrane integrity (PMI), mitochondrial function, apoptotic and necrotic spermatozoa percentage, and sperm cells that generated NO were determined after 2, 24, and 48 h of storage. The preserved semen was also analysed for the activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione (GSH) content, and malondialdehyde (MDA) production. Taurine, in both doses, may improve the antioxidant status of stored turkey semen as well as sperm motility, viability and functionality regardless of ejaculate quality, as manifested by increased SOD and CAT activities, reduced MDA levels, and enhanced sperm characteristics, i.e. plasma membrane integrity (PMI), mitochondrial membrane potential (MMP), total motility (TMOT), and progressive motility (PMOT). In turn, the addition of EGT increased GSH content in the external environment and suppressed lipid peroxidation in turkey spermatozoa, in particular those from low-quality (LQ) ejaculates. However, it appears that EGT could deliver more satisfactory results when added at lower concentrations.

Key words: taurine, ergothioneine, turkey semen, liquid preservation, antioxidants

In recent decades, numerous research efforts have been made to improve the preservation of turkey semen in a liquid state for up to 24 hours. The viability and fertilising capacity of turkey spermatozoa are compromised in both undiluted and diluted states (Lake and Ravie, 1982). Decreased sperm motility reduces fertility (Douard et al., 2000). High semen quality is difficult to maintain even for 24 hours (Thurston et al., 1994).

The evaluation of semen quality provides information about the male reproductive potential and is a major predictor of fertility and the subsequent hatchability of eggs (Mohan et al., 2018). The fertilising capacity of ejaculates can be predicted based on quality parameters such as semen volume, sperm motility, sperm concentration, pH, and colour (Mussa et al., 2023). In turkeys, the yellow colour of semen is associated, among others, with large numbers of abnormal spermatozoa (Thurston et al., 1975), whereas the grey colour is associated with a lower sperm count. The quality of a single ejaculate may be determined based on selected quantitative and qualitative parameters. Sperm must be motile to pass the reproductive tract and enter the sperm storage tubules of the female (Bakst et al., 1994). Semen derived from toms producing highly motile spermatozoa is more likely to reach and occupy these storage sites than the semen of toms characterised by low sperm motility (Holsberger et al., 1998). Semen of lower quality contains large numbers of abnormal spermatozoa, spermatids, and spermiophages (Thurston et al., 1975), and is characterised by higher activities of aspartate aminotransaminase, acid phosphatase, and superoxide dismutase (SOD), as well as higher total protein content (Hess and Thurston, 1984; Słowińska et al., 2011).

The antioxidant system found in semen, comprising enzymes and natural antioxidants, serves to inhibit or minimise the formation and spread of peroxides (Partyka et al., 2012). The initial inhibition of reactive oxygen species (ROS) involves SOD, glutathione peroxidase (GPx) and metal-binding proteins. The subsequent suppression of ROS generation relies on natural antioxidants such as vitamins A, C, E, uric acid, glutathione (GSH), and carotenoids (Surai et al., 1998). In avian semen, the

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balance between ROS and antioxidants plays a crucial role in maintaining plasma membrane integrity (PMI), sperm viability, and fertilising capability (Khan, 2011). For example, impaired SOD activity may intensify lipid peroxidation, thus decreasing semen quality (Froman and Thurston, 1981). In turn, GPx provides protection against lipid peroxides in sperm plasma membranes by suppressing the chain reaction of lipid peroxidation (Khan, 2011). High levels of GSH in seminal plasma seem to play a role in both protection against oxidative damage and improvement in sperm motility and morphology. A decrease in GSH content is associated with a decline in semen quality and fertility (Ansari et al., 2021). On the other hand, the presence of malondialdehyde (MDA) in semen is indicative of lipid peroxidation. Lipid peroxidation triggers the loss of PMI, and hence increases cell permeability, inactivates enzymes, causes structural damage to DNA, and finally cell death (Halliwell, 1994).

The role of taurine (TAU) and ergothioneine (EGT) supplementation in the liquid preservation of high- and low-quality (HQ and LQ, respectively) turkey ejaculates over 48 hours has not been investigated to date. Various concentrations of TAU have been used for several-hour storage of turkey sperm (Slanina et al., 2018) and cryopreservation of rooster semen (Partyka et al., 2017). However, the efficacy of EGT was tested only in cryopreserved rooster semen (Thananurak et al., 2020), and the obtained results were inconclusive. We hypothesised that certain levels of TAU and EGT may enhance the characteristics of turkey ejaculates. Therefore, the aim of this study was to determine whether TAU and ERG additives can improve the parameters of HQ and LQ turkey semen during prolonged liquid preservation.

Material and methods

Animals and semen collection

Semen samples (n=40) were obtained from adult male turkeys of the Big 6 line (Aviagen Turkeys Ltd., Chowley, Cheshire, UK). The turkeys were kept on a breeding farm (GERCZAK Nord-Pol Hatchery, Iława, Poland) in Kozia Góra (Warmian-Masurian Voivodeship, Poland) which observes rigorous hygienic practices and nutritional requirements. All samples were collected during a four-week period. All turkeys were approximately 41 weeks old at the beginning of the experiment. Once a week, 10 ejaculates (5 of high quality and 5 of low quality) were obtained by abdominal massage from ten different toms (Burrows and Quinns, 1937). Every week, ten other males were chosen for semen collection. Sperm samples were taken from individuals and they were not pooled. The toms that produced LQ semen were separated from those producing HQ semen. All ejaculates were collected with disposable syringes and diluted with the Extendyl extender (IMV Technologies, L'Aigle, France) in a 1:2 ratio (semen v/extender v).

Before dilution, the ejaculates were divided into two quality groups based on their colour. Ejaculates characterised by a white, milky, or pearl colour and a thick consistency were placed in the high-quality group (HQ = 20), whereas ejaculates characterised by a yellow, yellowish, or grey colour and a watery consistency were placed in the low-quality group (LQ = 20). Semen colour was examined against a blue background by a qualified farm employee, and each ejaculate was assigned to one of the two quality groups. The quality of each ejaculate was later confirmed in a laboratory by measuring the total protein content of seminal plasma (Thurston et al., 1982) using the Bradford method (1976). Immediately after collection, the ejaculates were placed in a heat-retaining thermobox (temperature of around 39°C) and transported to the laboratory to preserve sperm viability.

Semen preparation

In the first step, sperm counts in each ejaculate sample were determined with the use of a light microscope and a Bürker counting chamber (Equimed-Medical Instruments, Kraków, Poland). Before evaluation, an aliquot of each ejaculate was diluted with 0.85% NaCl to a final ratio of 1:800. The total number of spermatozoa was determined in twenty squares of the Bürker chamber, and the average number of sperm cells per square was calculated.

In the next step, every fresh ejaculate was stirred and divided by volume between three laboratory tubes. The first part, containing approximately ¼ of fresh ejaculate, was subjected to double centrifugation at 10000 × g for 10 minutes in order to obtain seminal plasma. The collected sperm pellets were washed twice with clear 0.85% NaCl, centrifuged at 10000 × g for 5 minutes each time, and then diluted with TBS containing 1% SDS (50 mM TRIS, 150 mM NaCl, 1% SDS; pH 7.5). The second part, containing approximately ½ of fresh ejaculate, was diluted with the extender to 200×10^9 spermatozoa/mL and divided into five equal fractions (control sample and four experimental samples). The fractions were placed in test tubes containing different amounts of TAU or EGT (5 mM or 10 mM). Semen fractions were stored at 5°C in a refrigerator. After 2, 24 and 48 h of storage, the semen was gently stirred and assessed for the analysed properties. For the analysis of biochemical parameters, the semen was centrifuged and separated into extracellular medium (referred to as the milieu) and sperm pellet (which was used to prepare sperm extract). The same procedure was applied to unpreserved semen. The remaining fraction of the fresh ejaculate was analysed for sperm motility, PMI, mitochondrial membrane potential (MMP), nitric oxide (NO) production by sperm, and detection of proapoptotic sperm.

Samples for the determination of biochemical parameters, obtained from fresh and preserved semen, were finally frozen at −80°C for further analyses. After thawing, they were tested for antioxidant enzyme activities, GSH content, and MDA levels. All biochemical parameters were measured in duplicate.

Analysis of sperm quality

Sperm motility

Semen was diluted with a motility buffer (50 mM of Tris, 120 mM of NaCl, 10 mM of glucose, 2 mM of $CaCl_2$, 0.5% BSA; pH 7.4) and heated in a dry block thermostat (TDB-120, Biosan, Poland) at 39°C for 15 min to activate sperm cells. Approximately 10 µL of warmed semen was spotted on a sperm counting plate. The following sperm kinetics parameters were evaluated with the use of a computer-assisted sperm analyser (CASA, Hamilton–Thorne Biosciences, IVOS version 12.3, USA): total motility (TMOT, %), progressive motility (PMOT, %), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), linearity coefficient (LIN, %), and straightness (STR, %). The following CASA settings were applied (according to the Hamilton Thorne technical guide v. 12.3 for turkey spermatozoa): frames acquired – 60, frame rate – 60 Hz, minimum cell contrast – 35, minimum cell size – 5 pixels, VAP threshold – 50 µ/s, STR threshold – 80.0%, VAP cutoff – 30.0 µ/s, and VSL cutoff – 15.0 µ/s.

Plasma membrane integrity

PMI was assessed using SYBR-14 and propidium iodide fluorescent dyes (Live/Dead Sperm Viability Kit; Molecular Probes, Eugene, OR, USA) as proposed by Fraser et al. (2002). Sperm samples were diluted with HEPES-buffered saline solution (130 mM of NaCl, 4 mM of KCl, 14 mM of fructose, 10 mM of HEPES, 1 mM of CaCl, 0.5 mM of MgCl, 0.1% BSA) to a final volume of 100 µl (30×10^6 spermatozoa/mL). The samples were first incubated at 39°C with 1 µl of SYBR-14 solution (1 mM of SYBR-14 in DMSO) for 10 min, and then with 1 µl of PI solution (2.4 µM of PI in Tyrode salt solution) for another 10 min. A minimum of 200 cells per slide were examined in each aliquot under an epifluorescence microscope (Olympus CH 30 RF-200, Tokyo, Japan). Membrane-intact spermatozoa exhibited green fluorescence and were classified as viable, whereas spermatozoa with damaged membranes exhibited red fluorescence and were classified as dead. The results were presented as the percentage (%) of sperm cells with intact plasma membranes.

Mitochondrial membrane potential

Semen samples were assessed for MMP using JC-1 and propidium iodide fluorescent dyes (Molecular Probes, Eugene, USA) according to the method described by Thomas et al. (1998). Sperm samples were diluted with HEPES-buffered saline solution using the procedure described above. The samples were first incubated at 39°C with 1 µL of JC-1 solution (1 mg of JC-1/mL in DMSO) for 15 min and then with PI (2.4 µM PI in Tyrode salt solution) for 5 min. At least 200 cells per slide were examined in each aliquot under an epifluorescence microscope (Olympus CH 30 RF-200, Tokyo, Japan). Sperm cells exhibiting orange fluorescence in the mid-piece region were classified as spermatozoa with active mitochondria and high MMP; sperm cells exhibiting green fluorescence were classified as spermatozoa with low MMP, whereas those with red fluorescence were classified as dead. A minimum of 200 spermatozoa were assessed per slide under an epifluorescence microscope (Olympus CH 30 RF-200, Tokyo, Japan) at 600× magnification. The results were presented as the percentage (%) of sperm cells with high MMP.

Nitric oxide production

The percentage of sperm cells generating NO was determined with DAF-2DA fluorescent dye, according to the method proposed by Lampiao et al. (2006). Sperm samples were diluted to 30×10^6 spermatozoa/mL with HEPES-buffered saline solution using the procedure described above. Spermatozoa were incubated at 39°C with 100 µL of DAF-2DA solution (20 µM DAF-2DA in PBS) in the dark for 120 min. Nitric oxide-producing spermatozoa were identified based on the presence of blue-green fluorescence in any segment (head, mid-piece, or tail). A minimum of 200 cells per slide were examined in each aliquot under light and epifluorescence microscopes. The results were presented as the percentage (%) of sperm cells producing NO.

Apoptotic-like and necrotic sperm

The percentage of sperm cells with proapoptotic and necrotic changes was validated with the use of the Vybrant Apoptosis Assay Kit #4 (Molecular Probes Inc., Eugene, OR, USA) according to the method described by Trzcińska and Bryła (2015), with some modifications. Sperm samples of 100 µl (30×10^6 spermatozoa/mL) were combined with 1 µl of JC-1 solution (as described above) and incubated at 39°C for 10 min to identify all spermatozoa (Dziekońska et al., 2022). Then, 1 µL of YO-PRO-1 (100 µM) was added to each sample, and the samples were kept at 39°C for 10 min. Finally, 1 µL of PI (2 µM) was added, and the samples were incubated at 39°C for 5 min. YO-PRO-1/JC-1/PI staining revealed three spermatozoa subpopulations: live sperm with intact membranes (orange fluorescence; YOPRO−/JC-1+/PI−), apoptotic sperm positive for YO-PRO-1 (green fluorescence; YOPRO+/JC-1+/PI−), and necrotic sperm positive for PI (red fluorescence; YOPRO−/JC-1−/PI+). A minimum of 200 spermatozoa were assessed per slide under an epifluorescence microscope. The results were presented as the percentage (%) of live, apoptotic, and dead spermatozoa.

The activity of antioxidant enzymes

The activity of antioxidant enzymes was measured both in the milieu and spermatozoa derived from each ejaculate. Enzyme activity was expressed in international units per millilitre (U/mL) or litre (U/L) or converted to spermatozoa concentration ($U/10^9$ sperm).

Superoxide dismutase activity

SOD activity was measured with the Ransod kit (Randox Laboratories, Crumlin, United Kingdom) according to the manufacturer's instructions. In this test, xanthine and xanthine oxidase generate superoxide anion radicals. These radicals react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to produce red formazan. The resulting colour corresponds to SOD activity, which is measured spectrophotometrically at 505 nm. One unit of SOD inhibits INT reduction by 50% per minute at 37°C.

Glutathione peroxidase activity

GPx activity was assessed with the Ransel kit (RandoxLaboratories, Ltd., London, UK) according to the manufacturer's instructions. The test is based on the ability of GPx to catalyse the oxidation of GSH by cumene hydroperoxide. Simultaneously, glutathione disulfide (GSSG) is converted to GSH, and NADPH is oxidised to $NADP^+$. The resulting decrease in absorbance is measured at 340 nm at 37°C (pH 7.2).

Catalase activity

CAT activity was measured using the Catalase Assay Kit (Merck Life Science, Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. Catalase easily decomposes H_2O_2 as a substrate. The remaining H_2O_2 reacts with quinoneimine dye to form a coupling product which is measured spectrophotometrically at 520 nm. One unit of CAT decomposes l M H_2O_2 per minute at a substrate concentration of 50 mM H_2O_2 at 25°C (pH 7.0).

Glutathione content

Total GSH concentration was estimated using the Bioxytech GSH-400 kit (Aoxre Bio-Sciences, CA, USA) according to the provided protocol. In the first step, substitution products (thioethers) are formed between a patented reagent, R1 (4-chloro-1-methyl-7-trifluromethylquinolinium methylsulfate) and the mercaptans (RSH) present in the sample. Thioethers are transformed into chromophoric thione in a reaction mediated by reagent R2 (30% NaOH). The obtained product has a maximal absorbance wavelength at 400 nm at 25°C. Glutathione concentration was reported in mM.

Malondialdehyde content

The content of MDA in semen was measured using the MDA-586 Assay Kit (Aoxre Bio-Sciences, CA, USA) according to the manufacturer's instructions. The assay is based on the reaction of N-methyl-2-phenylindole (R1, NMPI) with MDA at 45°C. One molecule of MDA reacts with 2 molecules of NMPI to yield a stable carbocyanine dye. The concentration of MDA in a sample is determined from the absorbance of the probe and the standard curve at 586 nm in the MDA-586 assay. The results were expressed in μmol MDA/mL or $\mu M/10^9$ sperm.

Statistical analysis

All data were analysed in the Statistica program (version 14.1.0, StatSoft, Poland). The results were expressed as means ± standard error of the mean (SEM). Selected sperm parameters were compared by one-way and two-way ANOVA with Tukey's post-hoc HSD test at a significance level of $P \leq 0.05$. Residuals for each model were assessed for both homogeneity of variance and adherence to a normal distribution. The data that were not normally distributed were transformed accordingly.

Results

The impact of TAU and EGT additives on the motility parameters of preserved turkey spermatozoa of high and low quality

The analysed additives affected the mobility and biological properties of preserved spermatozoa. Generally, 5 mM TAU improved sperm motility parameters during semen storage ($P \leq 0.05$). In HQ ejaculates, TAU increased TMOT throughout storage, VAP after 24 h, and BCF after 48 h of preservation (Table 1). In LQ ejaculates, TAU induced a significant improvement not only in TMOT, but also in PMOT, VAP and VCL (2 h), STR and LIN (24 h) as well as VSL and BCF (48 h) (Table 1). Ergothioneine induced less significant changes in the motility characteristics of sperm, and its effects were most pronounced when it was added at lower concentrations (Table 1).

The impact of TAU and EGT additives on the functional parameters of preserved turkey spermatozoa of high and low quality

Both additives increased PMI, MMP, and NO formation in cells. It should be noted that the percentage of NO-producing spermatozoa was higher in LQ ejaculates after the treatment with additives (Table 2). The percentage of apoptotic sperm was limited in ejaculates of both high and low quality treated with either TAU or EGT but only for the first two hours of storage ($P \leq 0.05$) (Table 2).

The impact of TAU and EGT additives on the antioxidant status of preserved turkey spermatozoa of high and low quality

The supplementation also induced changes in the antioxidant potential of spermatozoa. Even though the activity of SOD in cells remained unaffected throughout storage, the activity of GPx has changed. The highest activity of the latter enzyme was observed in all control samples ($P \leq 0.05$). Both additives limited GPx activity, but TAU exerted a more significant effect (Table 3). Greater fluctuations were observed in CAT activity in sperm. It should be noted that CAT activity was much lower in cells derived from HQ than LQ ejaculates, regardless of the type of additive. In contrast, MDA levels were lower in sperm from LQ ejaculates (Table 3).

Table 1. Motility parameters of preserved (T5, T10, E5, E10, C) spermatozoa from high- (HQ) and low-quality (LQ) turkey ejaculates

Time (h)	Quality	Sample	TMOT (%)	PMOT (%)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
2	HQ	T5	50.6±4.5 a	29.3±5.4	79.3±3.2	73.8±3.6	95.5±3.6	2.9±0.1	12.5±2.5	92.0±2.0	85.7±2.6
		T10	38.6±4.3 ab	37.4±5.3 x	79.0±3.5	75.5±3.6	95.1±3.3	2.5±0.2	13.1±2.5	95.1±0.9	80.0±2.3
		E5	40.0±5.1 ab	33.8±8.3	83.2±3.5	77.8±3.6	100.2±3.2	2.9±0.2	11.8±2.0	92.9±1.5	78.9±2.5
		E10	35.8±4.9 b	28.5±8.7	79.7±3.2	76.0±3.6	93.3±3.2	2.5±0.2	14.7±3.1	94.4±1.3	81.9±2.3
		C	38.5±4.6 ab	15.5±4.3	80.5±3.9	74.9±4.4	97.6±3.9 x	2.5±0.2	12.5±2.3	92.9±1.9	78.6±3.0
	LQ	T5	40.4±3.9 a	31.0±6.4 a	84.7±3.8 a	79.8±4.1	101.6±3.7 a	2.6±0.2	13.5±2.6	93.7±1.5	83.8±5.0
		T10	31.8±4.0 b	18.6±5.3 by	82.6±3.2 ab	77.2±3.4	97.6±3.8 a	2.6±0.2	16.1±3.5	93.4±2.2	81.5±6.3
		E5	38.4±4.9 a	27.0±8.9 ab	79.5±2.6 ab	71.5±2.7	94.6±3.9 a	2.7±0.3	12.3±2.1	92.9±1.3	81.8±1.8
		E10	38.9±3.5 a	29.2±7.5 ab	79.5±3.3 abx	74.4±3.5	97.1±3.9 a	2.7±0.1	14.2±2.8	93.3±1.5	76.5±9.5
		C	30.2±3.6 b	18.8±3.7 b	75.0±3.4 b	71.2±3.6	90.7±3.8 by	2.4±0.2	11.8±2.5	94.1±1.0	76.3±6.9
24	HQ	T5	38.0±3.8 a	23.5±6.7	89.7±4.2 a	72.3±3.8	96.0±4.9	2.4±0.2	13.0±2.6	92.5±1.2	77.0±2.4
		T10	26.9±3.8 abc	14.3±6.8	82.9±5.7 ab	68.1±3.4	89.5±3.4	2.6±0.2	13.3±2.1	93.4±1.0	76.1±2.0
		E5	21.8±3.7 abc	19.9±3.5	77.7±9.4 ab	73.4±3.6	94.0±4.0	2.6±0.3	10.0±1.8	93.8±1.0	79.7±2.2
		E10	18.0±3.5 c	14.0±5.2	69.8±4.2 by	69.7±3.3	93.0±4.8	2.5±0.2	14.9±2.6	94.5±1.0	77.6±2.5
		C	29.9±3.7 bx	15.9±6.9	71.5±5.8 ab	69.7±3.7	91.6±3.8	3.0±0.3	11.2±1.7	92.9±1.5	77.6±2.6
	LQ	T5	32.9±3.9 a	20.3±3.4 a	74.7±11.5	76.7±3.9	94.2±4.2	2.3±0.2	10.9±2.1	94.2±1.2 ab	83.1±2.3 a
		T10	26.0±3.6 ab	15.0±2.1 abc	85.6±8.6	75.2±4.1	90.1±4.1	2.0±0.2	14.5±2.1	96.2±0.4 a	83.2±1.7 a
		E5	26.0±3.7 ab	15.5±3.3 abc	66.7±8.8	69.9±3.1	90.3±3.0	2.5±0.1	12.1±2.6	94.6±1.0 ab	77.6±2.2 ab
		E10	20.3±3.1 ab	11.7±2.5 b	70.8±8.2	68.7±3.7	93.2±3.9	2.4±0.2	15.2±3.0	91.6±2.5 b	75.0±3.0 b
		C	17.8±2.5 by	9.7±1.6 c	91.8±15.3	74.6±5.1	91.3±5.1	2.0±0.2	11.6±2.4	93.3±0.6 ab	81.1±1.8 ab
48	HQ	T5	18.8±3.3	9.9±2.3 y	77.9±14.3	73.7±4.7	88.8±4.8	2.2±0.2	8.0±2.2 b	95.2±1.2	83.8±2.6
		T10	17.2±2.9	10.8±2.0	79.2±13.5	78.8±3.8	97.6±3.0	2.2±0.2	15.5±3.4 a	93.8±1.6	81.1±2.4
		E5	14.6±2.7	8.6±2.2	69.6±4.1 y	72.1±4.0	90.0±4.3	2.4±0.2	14.1±2.6 ab	93.9±1.3	80.0±2.4
		E10	13.8±2.3	7.8±1.9	70.3±10.8	69.9±3.8	93.0±6.9	2.2±0.2	11.1±2.4 ab	94.6±1.1	78.0±3.0
		C	13.6±2.8	9.9±2.1	65.4±4.3	71.5±4.0 y	89.7±4.1	2.5±0.2	11.9±2.1 ab	94.1±1.0	80.4±2.0
	LQ	T5	22.1±2.9 a	15.4±2.3 ax	77.4±4.9	71.7±4.3 ab	88.8±4.9	2.1±0.2	8.4±2.4 b	90.88±4.0	78.6±4.1
		T10	14.2±2.3 b	10.3±1.8 abc	77.8±10.3	72.3±5.0 ab	95.5±5.4	2.0±0.3	15.0±2.9 a	92.28±2.7	79.0±4.4
		E5	15.2±2.2 b	11.0±2.0 abc	94.3±8.5 x	73.7±4.1 ab	91.6±5.2	2.6±0.3	14.5±2.8 a	96.04±0.7	82.4±2.4
		E10	14.2±1.8 b	8.8±1.4 c	72.4±7.6	69.0±2.3 b	87.6±3.8	2.5±0.2	12.9±2.9 ab	95.48±0.8	81.0±1.8
		C	14.1±2.6 b	9.0±1.9 b	82.8±7.3	83.0±7.0 ax	101.7±7.8	2.6±0.3	10.3±2.1 ab	94.32±1.1	81.0±1.9

Different letters a, b, c, d show significant differences between various additives (P≤0.05) within the semen of selected quality.

Different letters x, y show significant differences between high (HQ) and low quality (LQ) turkey ejaculates (P≤0.05) at one measurement time.

TMOT – total motility, PMOT – progressive motility, VAP – average path velocity, VSL – straight line velocity, VCL – curvilinear velocity, ALH – amplitude of lateral head displacement, BCF – beat cross frequency, LIN – linearity coefficient, STR – straightness.

Table 2. Functional parameters of preserved (T5, T10, E5, E10, C) spermatozoa from high- (HQ) and low-quality (LQ) turkey ejaculates

Time (h)	Quality	Sample	PMI (%)	MMP (%)	NO (%)	Apoptotic spermatozoa (%)	Necrotic spermatozoa (%)
2	HQ	T5	85.8±1.6 a	80.5±3.4	80.5±1.6 a	18.4±0.6 b	4.6±0.2 b
		T10	79.2±3.6 ab	80.5±4.1	78.2±2.3 ab	16.7±1.3 b	3.7±0.3 bcy
		E5	81.1±3.2 ab	77.5±3.6	71.2±3.9 by	18.1±1.0 b	5.1±0.3 ac
		E10	80.6±3.4 abx	78.7±3.8	83.4±1.3 a	26.6±2.3 ax	5.5±0.9 ac
		C	75.0±4.3 b	78.3±3.6	72.9±3.2 b	19.0±0.9 b	2.6±0.3 cy
	LQ	T5	83.8±1.1 ab	81.9±2.7	75.8±5.6 ab	18.9±1.2 b	5.4±0.3 b
		T10	89.6±2.1 ax	87.9±4.0	79.7±2.5 ab	23.2±1.3 a	6.0±0.8 abx
		E5	86.1±1.6 a	80.5±3.2	85.2±1.9 abx	15.6±0.4 c	6.8±0.5 a
		E10	74.8±3.3 by	78.5±4.1	85.9±2.4 a	17.5±1.1 by	4.9±0.2 b
		C	78.1±3.9 b	75.3±4.6	72.6±6.9 b	20.7±1.5 ab	6.0±0.5 abx
24	HQ	T5	79.4±3.9 ab	77.1±2.2 a	55.8±4.8 a	39.7±2.0 b	4.1±0.3 b
		T10	77.8±4.5 ab	77.0±2.0 a	55.5±4.9 ay	42.4±2.9 b	7.0±0.6 a
		E5	79.9±4.7 a	68.3±2.0 ab	48.7±4.4 by	41.3±2.6 b	5.5±0.6 ab
		E10	77.7±3.6 ab	63.2±3.5 by	46.7±4.1 aby	50.5±3.0 ax	4.9±0.7 b
		C	72.6±2.9 b	64.0±2.1 b	50.0±4.3 ab	44.9±2.9 ab	4.4±0.3 b
	LQ	T5	76.9±1.4	74.8±2.0	61.0±4.3 a	35.3±1.9	4.4±1.1 ab
		T10	79.6±1.5	74.3±2.3	64.2±2.8 ax	37.9±2.9	5.2±0.5 a
		E5	74.9±1.9	70.2±2.7	60.3±3.3 abx	38.5±2.3	3.4±0.3 b
		E10	75.2±1.7	70.9±1.9 x	60.5±2.3 abx	39.1±3.1 y	4.2±0.7 ab
		C	76.1±2.0	73.4±2.4	51.5±3.5 b	38.8±2.0	5.4±0.4 a
48	HQ	T5	66.5±1.9 ab	64.4±1.9 ay	58.1±4.9 ab	46.0±1.4 abcx	2.4±0.6 a
		T10	68.9±2.0 a	66.0±2.1 a	61.9±4.1 a	49.9±1.7 ax	1.6±0.2 aby
		E5	69.3±2.0 ax	60.5±3.2 a	52.8±3.9 aby	42.2±2.1 bc	0.5±0.1 by
		E10	69.1±1.8 ax	59.4±3.6 b	51.7±2.6 aby	46.9±1.8 abc	1.4±0.2 ab
		C	60.8±3.3 b	62.9±3.5 a	47.7±3.9 by	36.9±1.7 c	2.6±0.6 a
	LQ	T5	64.2±1.8 ab	67.5±1.9 ax	56.6±2.5 b	34.1±1.4 by	2.1±0.3 by
		T10	65.6±1.2 a	67.0±2.5 a	67.8±2.6 a	35.0±1.4 by	2.9±0.7 abx
		E5	62.7±2.2 aby	61.5±2.9 ab	59.7±3.2 abx	36.4±1.5 b	3.9±0.5 ax
		E10	61.7±2.2 aby	60.8±2.9 b	58.5±3.0 bx	44.5±3.2 a	1.9±0.4 b
		C	59.5±2.3 b	62.2±2.2 ab	58.5±4.4 bx	36.2±2.4 b	3.2±0.5 abx

Different letters a, b, c, d show significant differences between various additives (P≤0.05) within the semen of selected quality.
Different letters x, y show significant differences between high (HQ) and low quality (LQ) turkey ejaculates (P≤0.05) at one measurement time.
PMI – plasma membrane integrity, MMP – mitochondrial membrane potential, NO – the percentage of sperm that generated nitric oxide, Apoptotic spermatozoa – the percentage of sperm with proapoptotic changes, Necrotic spermatozoa – the percentage of sperm with necrotic changes.

Table 3. Antioxidant status of preserved (T5, T10, E5, E10, C) spermatozoa from high- (HQ) and low-quality (LQ) turkey ejaculates

Time (h)	Quality	Sample	SOD activity ($U/10^9$ sperm)	GPx activity ($U/10^9$ sperm)	CAT activity ($U/10^9$ sperm)	GSH ($mM/10^9$ sperm)	MDA ($\mu M/10^9$ sperm)
1	2	3	4	5	6	7	8
2	HQ	T5	6.2±0.8	0.7±0.4 bx	13.2±4.7 ay	5.2±0.6 a	2.9±0.5 ab
		T10	5.3±0.8	0.8±0.1 ab	13.5±6.3 a	3.7±1.1 b	1.5±0.7 ab
		E5	4.4±0.9	0.6±0.5 by	9.90±3.3 ab	5.9±0.4 a	0,8±0.5 b
		E10	6.3±0.8	0.7±0.2 ab	6.59±1.7 by	5.4±0.2 ab	4.8±3.0 a
		C	5.8±1.3	1.01±0.4 a	15.3±8.2 a	6.2±0.4 a	4.0±2.5 a
	LQ	T5	7.6±1.0	0.24±0.1 by	20.3±5.1 abx	6.1±0.5 a	3.9±1.1 a
		T10	5.2±0.8	0.74±0.1 ab	15.9±5.3 b	4.1±0.6 b	0.7±0.2 b
		E5	6.0±0.8	0.83±0.1 abx	12.4±3.8 b	5.3±0.5 ab	0.7±0.2 ab
		E10	6.2±0.8	0.92±0.2 a	21.8±6.6 abx	5.3±0.7 ab	0.5±0.2 b
		C	5.4±1.5	1.11±0.4 a	30.3±8.9 a	6.7±0.8 a	4.6±2.1 ab

Table 3 – contd.

1	2	3	4	5	6	7	8
24	HQ	T5	6.8±1.3	0.47±0.2 c	9.2±2.6 by	5.9±0.5 ab	4.2±2.6
		T10	6.7±0.8	0.74±0.3 b	13.1±4.2 ab	5.1±0.4 ab	6.7±3.8
		E5	5.6±0.6	0.51±0.1 aby	12.2±3.3 aby	4.8±0.5 b	4.5±2.2
		E10	5.7±1.1	0.56±0.2 ab	12.9±4.6 ab	6.0±0.7 ab	6.2±3.7
		C	6.3±1.1	0.93±0.3 a	17.9±4.6 ay	6.6±0.7 a	5.8±4.3
	LQ	T5	6.7±1.1	0.42±0.1 b	21.6±7.2 x	5.2±0.8 ab	2.2±1.3
		T10	6.3±0.9	0.45±0.1 b	21.1±6.6	5.4±0.9 ab	4.5±2.5
		E5	4.8±0.8	0.88±0.1 abx	35.0±5.3 x	5.9±0.7 ab	3.1±2.1
		E10	5.2±1.1	0.84±0.2 ab	27.2±8.0	4.3±0.6 b	2.2±1.9
		C	5.7±1.2	0.98±0.1 a	30.3±6.9 x	6.6±0.3 a	5.8±3.1
48	HQ	T5	5.5±0.5	0.56±0.1 b	9.3±4.2 y	5.4±0.3	5.8±4.4
		T10	5.9±0.8	0.77±0.1 abx	11.9±5.9 y	5.4±0.5	6.4±3.9
		E5	6.2±0.9	0.60±0.2 b	12.5±7.1 x	6.2±0.2	5.3±3.3
		E10	4.9±0.9	0.69±0.2 aby	13.9±5.6	5.5±0.4	6.4±4.7
		C	6.3±0.3	1.48±0.3 a	9.2±2.8 y	6.4±0.6	6.7±4.9
	LQ	T5	5.7±1.0	0.55±0.2 b	23.9±7.6 ax	2.9±1.2 c	2.7±2.0
		T10	5.9±1.1	0.49±0.1 by	25.4±6.1 ax	3.5±1.3 abc	3.9±3.5
		E5	5.2±1.1	0.52±0.2 b	8.7±3.2 by	4.5±0.9 abc	6.6±4.5
		E10	6.1±0.5	0.98±0.2 abx	15.1±3.3 ab	3.6±0.8 abc	2.7±0.5
		C	6.2±0.7	1.10±0.2 a	26.3±5.8 ax	6.1±0.2 a	4.3±3.5

Different letters a, b, c, d show significant differences between various additives (P≤0.05) within the semen of selected quality.
Different letters x, y show significant differences between high (HQ) and low quality (LQ) turkey ejaculates (P≤0.05) at one measurement time.
SOD – superoxide dismutase, GPx – glutathione peroxidase, CAT – catalase, TAS – total antioxidant status, GSH – glutathione, MDA – malondialdehyde.

Table 4. Antioxidant status of preserved (T5, T10, E5, E10, C) milieus from high- (HQ) and low-quality (LQ) turkey ejaculates

Time (h)	Quality	Sample	SOD activity (U/mL)	GPx activity (U/mL)	CAT activity (U/mL/min)	GSH (mM)	MDA (μM)
1	2	3	4	5	6	7	8
2	HQ	T5	30.7±6.5	7.8±1.1 abx	186.8±58.1 a	5.0±1.5 b	5.1±0.9 ab
		T10	30.7±6.7	4.9±1.5 b	51.5±14.9 b	5.2±0.8 b	5.1±0.7 ab
		E5	24.7±5.2	8.8±2.4 ab	138.3±41.5 abx	17.5±2.7 a	5.9±1.5 ab
		E10	29.6±6.1	7.2±1.0 b	90.7±19.4 ab	22.7±1.21 a	1.7 ±0.7 b
		C	25.7±5.4	14.2±3.7 a	60.0±28.9 b	4.6±2.8 b	13.5± 7.1 a
	LQ	T5	21.7±4.0 ab	2.9±0.6 y	83.6±23.3	5.6±0.6 c	4.2± 0.6 ab
		T10	16.9±3.4 ab	6.2±2.1	51.6±16.9	5.7±1.5 c	5.1±0.7 ab
		E5	17.3±2.9 ab	4.9±0.9	28.4±12.8 y	14.3±2.5 b	5.6±1.7 ab
		E10	33.3±5.9 a	7.1±1.9	92.4±25.1	21.0±2.1 a	1.4±0.5 b
		C	14.3±4.0 b	5.8±2.1	85.3±41.8	3.6±1.0 c	9.9±5.5 a
24	HQ	T5	25.7±9.3 b	6.5±2.1	146.3±71.8 bc	4.4±1.1 b	8.5±6.9
		T10	27.4±6.9 ax	5.4±1.9	228.0±27.4 ax	4.3±1.5 b	6.9 ± 6.6
		E5	14.9±2.4 bc	9.1±1.9 x	108.7±15.9 c	26.2±7.2 ax	9.9±4.9
		E10	25.0±7.0 b	8.4±1.1 x	113.2±11.5 b	34.0±11.2 ax	5.7±1.1
		C	12.5±1.8 c	6.6±1.6	86.7±44.4 c	2.8±2.2 b	11.9±7.7
	LQ	T5	18.1±5.0	3.8±0.8	68.0±29.7	4.3±0.6 b	6.6±4.9
		T10	13.4±3.7 y	3.7±0.9	42.7±20.8 y	4.2±1.5 b	8.2±4.2
		E5	14.8±5.2	3.9±1.5 y	70.7±22.9	14.4±2.9 ay	7.3±2.9
		E10	19.7±5.9	3.8±1.6 y	61.3±25.5	19.1±2.8 ay	6.6±4.5
		C	11.6±2.5	6.8±2.1	45.3±14.5	3.5±1.4 b	9.2±4.5

Table 4 – contd.

Time (h)	Quality	Sample	SOD activity (U/mL)	GPx activity (U/mL)	CAT activity (U/mL/min)	GSH (mM)	MDA (μM)
1	2	3	4	5	6	7	8
48	HQ	T5	27.2±11.8	7.9±2.3	102.7±34.3 a	4.5±1.6 c	10.6±6.5
		T10	15.1±1.9	8.4±2.3	72.0±17.2 ab	4.5±1.1 c	8.8±6.3
		E5	17.8±2.0	7.9±0.3 x	106.7±8.7 a	28.6±8.6 bx	11.8±5.6
		E10	30.9±12.0	7.1±1.5 x	114.7±5.1 a	52.4±13.4 ax	8.5±5.2
		C	17.8±2.0	10.1±3.3	37.3±23.6 b	4.3±1.5 c	13.1±7.3
	LQ	T5	17.3±3.8	4.3±1.4	98.7±27.9	3.4±0.9 b	7.9±4.8
		T10	17.8±4.5	5.1±1.0	77.3±26.1	3.7±1.2 b	7.7±5.6
		E5	17.0±4.0	4.2±1.1 y	68.0±15.3	19.5±1.2 ay	9.1±3.5
		E10	16.3±3.8	4.5±1.8 y	105.3±27.7	23.8±3.9 ay	8.2±4.6
		C	14.2±5.4	5.9±3.2	100.0±25.2	3.2±1.7 b	11.0±5.6

Different letters a, b, c, d show significant differences between various additives (P≤0.05) within the semen of selected quality.

Different letters x, y show significant differences between high (HQ) and low quality (LQ) turkey ejaculates (P≤0.05) at one measurement time.

SOD – superoxide dismutase, GPx – glutathione peroxidase, CAT – catalase, TAS – total antioxidant status, GSH – glutathione, MDA – malondialdehyde.

Table 5. ANOVA sources of variations in parameters of high-quality (HQ) turkey semen

	Parameters	Time of storage		Treatment		Time of storage × Treatment	
		F-value	p-value	F-value	p-value	F-value	p-value
Milieu	SOD activity	0.992	0.379	1.081	0.377	0.194	0.990
	GPx activity	1.040	0.362	0.365	0.832	0.194	0.990
	CAT activity	1.897	0.162	1.092	0.372	0.858	0.558
	GSH content	2.846	0.069	48.997*	0.000*	1.822	0.098
	MDA level	1.423	0.252	0.552	0.698	0.145	0.996
Spermatozoa	TMOT	10.387*	0.000*	0.401	0.807	0.291	0.965
	PMOT	6.018*	0.005*	0.151	0.961	0.630	0.748
	VAP	1.070	0.352	1.010	0.412	0.553	0.810
	VSL	0.943	0.397	0.585	0.675	0.305	0.960
	VCL	1.229	0.302	1.064	0.385	0.853	0.563
	ALH	4.540*	0.016*	0.424	0.791	1.056	0.410
	BCF	0.119	0.888	0.238	0.915	0.584	0.786
	STR	0.022	0.978	0.532	0.713	0.497	0.852
	LIN	0.083	0.920	0.534	0.711	0.227	0.984
	PMI	6.549*	0.003*	0.387	0.817	0.290	0.966
	MMP	0.453	0.639	1.742	0.157	0.901	0.524
	NO	1.421*	0.711	0.160	0.040	0.196	0.025
	Apoptotic	29.803*	0.000*	1.884	0.130	0.490	0.857
	Necrotic	23.245*	0.000*	0.706	0.592	1.430	0.210
	SOD activity	1.417	0.253	0.336	0.852	0.266	0.974
	GPx activity	0.404	0.670	2.372	0.066	0.920	0.509
	CAT activity	0.924	0.404	1.080	0.378	0.807	0.600
	GSH content	0.832	0.442	1.387	0.254	1.348	0.245
	MDA level	0.113	0.894	0.528	0.716	0.126	0.998

Data marked with asterisks (*) are statistically significant (P≤0.05).

SOD – superoxide dismutase, GPx – glutathione peroxidase, CAT – catalase, TAS – total antioxidant status, GSH – glutathione, MDA – malondialdehyde, TMOT – total motility, PMOT – progressive motility, VAP – average path velocity, VSL – straight line velocity, VCL – curvilinear velocity, ALH – amplitude of lateral head displacement, BCF – beat cross frequency, STR – straightness, LIN – linearity coefficient, PMI – plasma membrane integrity, MMP – mitochondrial membrane potential, NO – the percentage of sperm that generated nitric oxide, Apoptotic spermatozoa – the percentage of sperm with proapoptotic changes, Necrotic spermatozoa – the percentage of sperm with necrotic changes.

The impact of TAU and EGT additives on the antioxidant status of preserved turkey milieus of high and low quality

Superoxide dismutase activity was significantly higher in samples with additives in HQ ejaculates after 24 h and in LQ ejaculates after 2 h than in controls (Table 4). However, all controls were characterised by lower SOD activity throughout storage (non-significant differences). The activity of GPx was significantly lower in samples with additives in HQ ejaculates after two hours of treatment. The reduction in GPx activity during semen storage was more pronounced in LQ ejaculates stored with EGT than in HQ ejaculates with this additive (Table 4). High-quality ejaculates preserved with either TAU or EGT were also characterised by higher CAT activity than control samples (Table 4). Glutathione content increased significantly throughout semen storage in EGT-treated samples, regardless of ejaculate quality, but the noted increase was more pronounced in HQ ejaculates (Table 4). Malondialdehyde content decreased significantly in HQ and LQ ejaculates preserved with TAU or EGT for 2 h (Table 4). A minor (non-significant) decrease in MDA concentration in samples with additives was observed throughout storage.

The summary of the ANOVA analysis

The summary of the ANOVA analysis for the high-quality turkey semen is presented in Table 5. The ANOVA revealed that the time of storage significantly influenced TMOT, PMOT, ALH and PMI in HQ sperm, and the percentage of apoptotic-like, necrotic and NO-producing cells. Moreover, the treatment affected the GSH content in the surrounding milieu. In contrast, both variables altogether did not affect any parameters of high-quality turkey semen.

The summary of the ANOVA analysis for the low-quality turkey semen is presented in Table 6. The time of storage significantly affected TMOT and PMOT in sperm, and like the above, the percentage of apoptotic-like, necrotic and NO-producing cells. Moreover, it also had a marked effect on the GSH content of sperm. The tested additives influenced the GSH content in the milieu, and GPx activity and GSH content in spermatozoa. The percentages of necrotic sperm were also affected by the additives. The ANOVA results demonstrated that time of storage × treatment were no sources of variations in any parameters of turkey semen of low quality.

Table 6. ANOVA sources of variations in parameters of low-quality (LQ) turkey semen

	Parameters	Time of storage		Treatment		Time of storage x Treatment	
		F-value	P-value	F-value	P-value	F-value	P-value
Milieu	SOD activity	3.159	0.052	1.847	0.136	0.376	0.928
	GPx activity	0.064	0.938	1.035	0.400	0.251	0.978
	CAT activity	2.123	0.131	0.395	0.811	0.315	0.956
	GSH content	0.889	0.418	23.364*	0.000*	0.217	0.986
	MDA level	0.792	0.459	1.395	0.251	0.259	0.976
Spermatozoa	TMOT	8.139*	0.001*	0.518	0.723	0.262	0.975
	PMOT	4.140*	0.022*	0.503	0.734	0.244	0.980
	VAP	0.440	0.646	0.218	0.927	1.908	0.082
	VSL	0.259	0.773	0.080	0.988	1.188	0.328
	VCL	2.377	0.104	1.056	0.389	1.944	0.076
	ALH	1.180	0.317	1.222	0.315	0.936	0.497
	BCF	0.294	0.747	0.811	0.525	0.909	0.518
	STR	0.261	0.771	0.601	0.664	0.557	0.807
	LIN	0.087	0.917	0.741	0.569	0.740	0.656
	PMI	2.613	0.084	0.937	0.451	1.954	0.075
	MMP	0.480	0.622	0.252	0.907	1.073	0.399
	NO	4.509*	0.016*	1.258	0.301	0.428	0.898
	Apoptotic	21.470*	0.000*	0.567	0.688	0.924	0.506
	Necrotic	12.368*	0.000*	2.666*	0.044*	1.833	0.096
	SOD activity	0.258	0.774	0.387	0.817	0.235	0.982
	GPx activity	0.212	0.810	7.434*	0.000*	0.982	0.463
	CAT activity	1.781	0.180	1.135	0.352	1.254	0.291
	GSH content	3.687*	0.033*	7.711*	0.000*	1.907	0.082
	MDA level	0.066	0.936	0.279	0.890	0.500	0.850

Data marked with asterisks (*) are statistically significant (P≤0.05).

SOD – superoxide dismutase, GPx – glutathione peroxidase, CAT – catalase, TAS – total antioxidant status, GSH – glutathione, MDA – malondialdehyde, TMOT – total motility, PMOT – progressive motility, VAP – average path velocity, VSL – straight line velocity, VCL –curvilinear velocity, ALH – amplitude of lateral head displacement, BCF – beat cross frequency, STR – straightness, LIN – linearity coefficient, PMI – plasma membrane integrity, MMP – mitochondrial membrane potential, NO – the percentage of sperm that generated nitric oxide, Apoptotic spermatozoa - the percentage of sperm with proapoptotic changes, Necrotic spermatozoa – the percentage of sperm with necrotic changes.

Discussion

Successful fertilisation depends primarily on sperm with high viability and motility (Salih et al., 2021). Unfortunately, a substantial part of preserved spermatozoa may be impaired due to oxidative stress that is associated with semen processing and storage (Sharma et al., 2015). The formation of peroxides during semen storage induces changes in sperm motility (de Lamirande and Gagnon, 1992) and decreases fertility (Ravie and Lake, 1985). Avian semen has a complex antioxidant system that relies on natural antioxidants and antioxidant enzymes, but these defence mechanisms may not be sufficient to overcome the effects of oxidative stress during preservation. The dilution procedure may trigger osmotic stress, which ultimately leads to oxidative stress within the semen (Partyka and Niżański, 2021). The presence of seminal plasma has been found to suppress spontaneous lipid peroxidation. It appears that the dilution of avian semen, which is necessary for short-term storage, reduces the antioxidant protection of spermatozoa. After 24 hours of storage, intracellular ROS levels usually exceed the available antioxidant capacity of spermatozoa (Słowińska et al., 2018).

Studies performed in selected mammals (Ijaz and Ducharme, 1995; Jang et al., 2006; Baran et al., 2009; Bucak et al., 2007; Dorado et al., 2014) and in turkeys (Slanina et al., 2018) have revealed that TAU prevents the loss of sperm motility and viability *in vitro*. This amino acid generally improves sperm viability and motility by enhancing epididymal antioxidant capacity, improving secretion activity, and maintaining the homeostasis of the epididymal microenvironment (Li et al., 2023). In the current study, TAU was significantly implicated in preserving the motility and viability of turkey sperm throughout storage, regardless of its quality. The values of TMOT were significantly improved, especially in samples treated with 5 mM TAU from HQ ejaculates. Interestingly, TAU induced a greater increase in PMOT in LQ ejaculates during preservation. The above could be attributed to the fact that TAU promotes changes in the osmotic pressure of the extracellular milieu. The intracellular accumulation of TAU primarily occurs through efficient uptake by a transport system (TauT protein) located in the plasma membrane. TauT plays a key role in regulating TAU concentration, sperm quality, and spermatogenesis in the testes and epididymides (Li et al., 2023). This transport system utilises the transmembrane gradients of Na^+ and Cl^- as the driving force (Kozlowski et al., 2008). In turkeys, the most abundant inorganic ions are Na^+ and Cl^- (Lake and Wishart, 1984). It is likely that in turkey ejaculates of low quality, TAU accelerated compensation of the ion concentration gradient. Sarıözkan et al. (2009) demonstrated that TAU not only exerts antioxidant effects, but also slows down cell apoptosis and regulates mitochondria. In the present study, TAU also decreased the percentage of apoptotic gametes in preserved turkey sperm, but the noted decrease was significant only for the first 24 hours. These observations could be attributed to the fact that TAU initially minimises energy loss and prevents a decrease in MMP. As a result, TAU decreases the susceptibility of membrane lipids to peroxidation, which enables sperm cells to produce energy. When added to the culture medium, TAU can improve sperm quality and function by stimulating mitochondrial energy metabolism (Du et al., 2019). Several studies have suggested that stimulation of NO production by eNOS may be initiated by TAU, and its depletion may impair both endothelium-derived NO production and NO activity (Guizoni et al., 2020). Since sperm motility is associated with both ATP levels and NO formation, turkey semen supplementation with TAU could enhance NO synthesis in turkey sperm and, in consequence, increase its motility. In turn, NO has been shown to enhance the long-term TauT expression and the TAU uptake by this transporter in epithelial cells through the cGMP pathway (Bridges et al., 2001). Taurine is synthesised in most mammals and birds (Surai et al., 2021). Although its presence was confirmed in rooster seminal plasma, it was not detected in turkey seminal plasma in earlier studies (Ahluwalia and Graham, 1966). However, there is a possibility that TAU appears in turkey semen only in trace quantities as it can be found in other tissues of this species (Spitze et al., 2003). However, no key enzymes for TAU synthesis have been identified in spermatozoa to date, thus spermatozoa have to rely on TauT to utilize extracellular TAU (Li et al., 2006). Recent research has shown that low extracellular TAU levels are associated with poor fertility (DasGupta et al., 2022). Moreover, Wu et al. (2022) suggested that the imbalance in TAU concentrations between spermatozoa and seminal plasma is associated with the sperm TauT activity. According to the cited authors, the downregulation of TauT protein and TAU deficiency could result in impaired sperm morphology, thus affecting early embryo development. This observation could explain why TAU was more effective in improving the motility of spermatozoa in LQ ejaculates: either the concentration of this amino acid in LQ turkey ejaculates is negligible or the key enzymes involved in the TAU biosynthesis pathway are less expressed or expressed hardly at all; or the addition of TAU to the external environment may initiate a more efficient action of TauTs caused by a higher level of emerging NO in LQ ejaculates. Nevertheless, further research is needed to confirm these hypotheses.

Taurine can also enhance sperm quality by inhibiting excessive ROS production in the mitochondria, suppressing lipid peroxidation and apoptosis (Yang et al., 2010). Taurine promotes the activity of antioxidant enzymes such as SOD (Higuchi et al., 2012), GPx (Nonaka et al., 2001), and CAT (Perumal et al., 2013). Partyka et al. (2012) detected the presence of CAT in chicken spermatozoa and seminal plasma. In the present study, CAT activity was observed in turkey semen, in both spermatozoa and the milieu, although it has not been reported previously. Taurine exerted varied effects on the activities

of antioxidant enzymes in turkey semen. This additive initially promoted CAT and SOD activities, but limited GPx activity. Increased activities of SOD and CAT in the extender are associated with an increase in the number of mRNA copies of SOD and CAT genes during semen storage with TAU. The pattern of GPx activity is different. Non-supplemented semen samples exhibit nearly fixed GPx levels in the long run, whereas in supplemented samples, GPx activity increased considerably with increasing chilling time (Bayomy et al., 2023). Turkey sperm necessitate a highly oxygenated environment during low-temperature *in vitro* storage (Sexton and Giesen, 1992). However, oxygen induces both the production of free radicals and impaired motility, which can be easily reversed by CAT activity (MacLeod, 1943). The reaction catalysed by CAT not only helps maintain normal ROS levels but also protects spermatozoa from potentially harmful ROS levels (Rubio-Riquelme et al., 2020). Although CAT activity increased significantly after the amino acid supplementation of semen in HQ ejaculates, it increased only slightly or remained unchanged in LQ semen. Therefore, it is likely that in LQ ejaculates, CAT expression or its catalytic efficacy may be limited as GSH content in the milieus derived from LQ and HQ ejaculates was comparable. Since CAT activity increased significantly in HQ ejaculates, the activities of other enzymes could decrease proportionally as H_2O_2 is regarded as the most cytotoxic oxygen metabolite in semen when present in large quantities (Rubio-Riquelme et al., 2020). Enhanced antioxidant potential of turkey semen was confirmed by lower MDA content in the milieus of both qualities. Interestingly, MDA content was significantly reduced only during the first two hours. Its further formation was also slightly limited but in a non-significant manner. This fact can be linked to enhanced CAT activity and the maintenance of GSH content in the environment, because MDA levels were highest in turkey semen preserved without any additives. Bayomy et al. (2023) reported a significant reduction of MDA content in stored rabbit semen supplemented with TAU and GSH. It appears that TAU acts on turkey semen mainly by both decreasing MDA content and restoring GSH levels. To the best of the authors' knowledge, this is the first study to examine GSH content in both fresh and stored turkey semen. Numerous studies have shown that TAU confers protection against oxidative stress by normalising GSH levels (Baliou et al., 2021). As demonstrated by Ansari et al. (2021), the presence of GSH in the extender improves sperm viability, PMI, acrosome integrity, motility, mitochondrial functionality, and fertility in Indian red jungle fowl through enhancing antioxidant potential and ameliorating oxidative stress. Taurine's effect on GSH can be attributed to the fact that cysteine is a precursor of both GSH and TAU molecules, and sparing cysteine content in the provision of TAU may lead to increased regeneration of GSH (Giustarini et al., 2023). The obtained results suggest that TAU may maintain GSH resources in the milieu, in particular that derived from HQ ejaculates.

Ergothioneine is a naturally occurring hydrophilic amino acid, a nutrient, and an antioxidant with a confirmed ability to scavenge ROS (Zullo et al., 2016). Ergothioneine is not widely used in short-term preservation of semen. Previous studies have demonstrated that EGT can increase the respiration of guinea pig spermatozoa (Hama et al., 1973) and improve the fertilisation rates of mouse ova (Mayumi et al., 1984). Salih et al. (2021) reported that EGT supplementation can improve both the TMOT and PMOT of cryopreserved rooster sperm. Çoyan et al. (2012) found that increasing levels of EGT (1, 2 and 4 mM) had positive effects on TMOT and PMOT and several kinetic parameters of ram sperm. In the current study, the 5 mM treatment induced a greater improvement in TMOT and PMOT, whereas the 10 mM treatment was more effective in enhancing the antioxidant capacity of the extracellular milieu. However, the fact that EGT promoted GSH formation in semen samples, especially those of high quality, deserves special attention. The biosynthesis of EGT could be highly correlated with GSH levels. The genes encoding GSH synthesis (gshA and gshB) could be involved in the synthesis of EGT and reversibly EGT could promote the synthesis of GSH through the Nrf2/ARE pathway (Narainsamy et al., 2016). In addition, numerous studies have demonstrated that EGT is linked to the GSH/GSSG redox couple and might promote GSH redox cycling (Oumari et al., 2019). This process could take place in turkey ejaculates, in particular in those preserved with a higher dose of EGT. As a result, the produced GSH could reverse the oxidation of EGT (Oumari et al., 2019). Thus, both antioxidants could interact to confer protection against oxidative stress, especially lipid peroxidation, as evidenced by the fact that MDA levels were lowest in turkey semen preserved with 10 mM EGT, in particular in LQ ejaculates. Ergothioneine's ability to stimulate fertilisation and prolong sperm viability (Hama et al., 1973) could be directly associated with its inhibitory effect on the formation of lipid peroxides. On the other hand, EGT content in seminal plasma of many mammals seems to be unrelated to GSH levels. In mammals, EGT may not be dependent on the redox cycle of GSH (Nikodemus et al., 2021). However, the present results indicate that the synthesis and regeneration of GSH might have utilised a metabolic pathway associated with the presence of EGT in the extracellular environment of turkey semen. According to Halliwell et al. (2018), EGT does not interfere with the critical roles of ROS/RNS in healthy tissues and acts only when oxidative stress is intensified. In the present study, the production of ROS probably began to increase on the first day of semen storage, which could explain why EGT action was more pronounced after 24 hours of storage. Ergothioneine improves PMI and mitochondrial activity, and it decreases the percentage of apoptotic and dead sperm cells (Salih et al., 2021). The percentage of turkey spermatozoa with intact plasma membranes slightly increased after EGT treatment, but mitochondrial functionality remained largely unaffected. It should be noted that in samples with 10 mM EGT, the percentage of apoptotic sperm even increased.

However, this observation could be attributed to the potentially harmful effect of a high EGT dose in the long run. Therefore, it is likely that EGT could deliver more satisfactory results when added at lower concentrations.

Conclusions

Taurine used as an additive during the liquid storage of turkey semen may modulate its antioxidant potential as well as sperm motility, viability and functionality. On the other hand, the addition of EGT to the semen extender may limit MDA formation and lead to GSH restoration. However, it appears that lower doses of EGT would produce better results in the prolonged preservation of turkey semen.

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Data availability statement

The data presented in this study are available upon request from the corresponding author.

Conflicts of interest

The authors declare no conflict of interest.

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