ORIGINAL ARTICLE

Veterinary Research Forum. 2026; 17 (1): 23 - 30
doi: 10.30466/vrf.2025.2051498.4640
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Sperm cryopreservation of sex-reversed rainbow trout (*Oncorhynchus mykiss*): incorporation of amino acids and anti-oxidants to the extender media to improve the anti-oxidant system

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Article Info

Article history:

Received: 30 January 2025
Accepted: 19 July 2025
Available online: 15 January 2026

Keywords:

Amino acid
Anti-oxidant
Rainbow trout
Sex-reversed
Sperm

Abstract

Anti-oxidants are vital for protecting sperm and can mitigate the negative effects associated with cryopreservation. This study was conducted to evaluate the protective effects of adding anti-oxidants (vitamins and amino acids) to extender in sex-reversed rainbow trout (*Oncorhynchus mykiss*) sperm. The collected sperm was diluted at the ratio of 1 : 5 by the extenders supplemented with different anti-oxidants including 1.00 mM of ascorbic acid and L-tryptophan, 2.00 mM of cysteine and α-tocopherol, and their combination. After dilution, the semen was aspirated into 0.50 mL straws, and the straws were placed on the tray, frozen for 10 min, and plunged into liquid nitrogen. Straws were thawed in a 30.00 ˚C water bath for 15 sec. The Sperm Class Analyzer System was used to evaluate sperm kinematics. The activity of anti-oxidant enzymes and lipid peroxidation were determined as oxidative stress indices. Our data indicated that the incorporation of anti-oxidants and amino acids increased sperm motility duration. The elevated activity of glutathione peroxidase, superoxide dismutase, and catalase in post-thaw samples indicates that the anti-oxidant system in sex-reversed rainbow trout sperm likely plays a crucial role in protecting membrane compounds from oxidation. In conclusion, the combination of 1.00 mM L-tryptophan and ascorbic acid to the extender media caused a prolonging effect in sperm motility after thawing and they have the potential to serve as effective agents for improving sperm cryosurvival.

Introduction

In breeding programs, sex-reversion or masculinization is utilized to produce single-sex populations both phenotypically and genetically in Salmonid fish.[1] Sex-reversed females, often called masculinized females or neomales, are genetically female but develop male characteristics, enabling them to produce sperm.[1,2] Due to the absence of sperm ducts, fish must be sacrificed to collect semen.[3,4] In aquaculture, the generation of exclusively female rainbow trout populations is highly valued, as they can reach marketable size rapidly before sexual maturity, maximizing profitability.[5]

The earliest study on cryopreservation of fish semen was conducted by Blaxter in 1953, enabling hybridization of Atlantic herring (*Clupea harengus*) populations that reproduce in different seasons.[6-7] Since the 1970s, cryo-preservation of fish sperm has been developed as a germplasm conservation technology. More than 200 fish species have been studied to develop semen preservation techniques, including approximately 40 marine species.[6-7] It is applicable to artificial reproduction, crossbreeding between species separated by temporal or geographical isolation, selective breeding based on family establishment, and gynogenesis induction.[8] The cryopreservation of sperm from sex-reversed rainbow trout requires additional investigation. Therefore, optimizing the cryopreservation extender medium is essential to overcome the limitations in artificial breeding. In this study, the types and concentrations of anti-oxidants in the cryopreservation extender were assessed to improve these outcomes.

Rainbow trout spermatozoa exhibit short-lasting motility, typically between 30 - 60 sec.[9] As a result, the cryopreservation process can negatively impact sperm quality and fertilization success.[10,11] Cryopreservation is also known to trigger the over-production of reactive oxygen species (ROS).[12,13] The ROS generation leads to oxidative stress in spermatozoa and can impair cellular functionality,

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axonemal structures, membrane stability and macromolecules, and nucleoprotein-DNA interactions, and damage mitochondrial mid-pieces and acrosomal reactions.[14-16] To mitigate and neutralize ROS-induced damage, anti-oxidants (enzymatic and non-enzymatic) play a crucial role.[17] Ascorbic acid (AA), commonly known as vitamin C, is a water-soluble, chain-breaking anti-oxidant involved in numerous biochemical reactions in both humans and animals.[18-19] L-tryptophan (L), an aromatic amino acid, is a structural component of proteins and possesses anti-oxidant properties.[20, 21] It has the ability to scavenge free radicals, protecting cells from oxidative damage.[22,23] Due to its various metabolic functions, L has been widely utilized in both research and clinical testing.[24] Alpha-tocopherol (T) is a natural anti-oxidant classified as a chain-breaking anti-oxidant. It works by scavenging free radicals, thereby interrupting radical reactions and stopping the propagation of chain reactions.[18] Cysteine (C), a sulfur-containing amino acid, neutralizes free radicals by directly interacting with them through chemical reactions.[25-27] The addition of anti-oxidants to the extender media can reduce oxidative damage.[28-31] Thus far, Martínez-Páramo *et al*.,[20] studied incorporation of AA and T to the freezing media to improve anti-oxidant system of cryopreserved European sea bass (*Dicentrarchus labrax*). Kocabaş *et al*.,[32] examined the effect of AA, L, and the combination of both on short-term sperm storage in rainbow trout (*Oncorhynchus mykiss*). To the best of our knowledge, no information is available about the effect of combination of different anti-oxidants on cryo-preservation of sex-reversed rainbow trout sperm until now. Within this context, the current study aimed to examine the improving impact of combinations of different anti-oxidants (AA-L, AA, C, and AA-T) on sperm motility parameters and oxidative stress indices of sex-reversed rainbow trout.

Materials and Methods

Masculinization and sample collection. The masculinization of sex-reversed females was conducted at the Ayta Production Facility, Rize, Türkiye, using 17α-methyltestosterone (Sigma-Aldrich, St. Louis, USA). Beginning in November 2021, these females received 17α-methyltestosterone through their feed at a dosage of 2.00 mg kg^{-1} of feed, maintained over a 60-day period at 10.00 ˚C. For evaluation of sex, juveniles before one year of age were euthanized. One gonad from each fish was removed and preserved in 70.00% ethanol. The entire length of the gonad was examined under a stereo dissection microscope for sex determination. Fish were classified as females if oocytes were visible, while those exhibiting both ovarian and testicular tissues were categorized as intersex. Fish under 1 year of age with developing gonads without visible oocytes at 40× magnification were identified as males. Throughout the spawning season, periodic checks were conducted on the fish by applying pressure to their abdomen to observe any release of eggs or milt when the fish approached 2 years of age. Once the spawning season ended, the remaining fish were euthanized, weighed, and dissected to measure gonad weight and identify their sex and stage of sexual maturation.[33] Milt was collected post-mortem by dissecting the testes and gently pressing them through double-layered gauze to remove any remaining testicular tissue from the individuals.[3,4] Sperm was collected from mature fish (n = 6).

Experimental procedure. Sperm samples with a motility rate of 70.00% were used for the experiments. The pooled semen was mixed with an immobilization medium at 1 : 5 ratio (semen : medium), containing 75.00 mM NaCl , 2.30 mM $NaHCO_3$, 1.50 mM $CaCl_2$, 0.40 mM $MgCl_2$, and 83.00 mM KCl, pH: 7.82, 10.00% dimethyl sulfoxide, and 10.00% egg yolk. All chemicals were obtained from Sigma-Aldrich. The diluted sperm was then distributed into separate 15.00 mL-Falcon tubes using a pipette for further treatment. The tested treatments are as follows: Treatment 1: Control (0.00 mM), AA (1.00 mM), L (1.00 mM), and AA (1.00 mM) + L (1.00 mM), Treatment 2: Control (0.00 mM), AA (1.00 mM), C (2.00 mM), and AA (1.00 mM) + C (2.00 mM), and Treatment 3: Control (0.00 mM), AA (1.00 mM), T (2.00 mM), and AA (1.00 mM) + T (2.00 mM). The selected AA,[8,31] T,[8,32] C[33] and L[8,34-36] concentrations were determined according to the results of our preliminary experiments and published studies.

Analysis of sperm quality parameters. A Sperm Class Analyzer System (Sperm Class Analyzer v. 4.0.0; Microptic S.L., Barcelona, Spain) paired with a phase-contrast microscope was utilized to assess sperm motility parameters. These parameters included curvilinear velocity (VCL; μm sec^{-1}), the speed along the actual movement trajectory, straightness (STR), velocity of the average path (VAP; μm sec^{-1}), straight-line velocity (VSL; μm sec^{-1}), the speed from the start to the end point along a straight line, and linearity of movement (LIN), the percentage ratio of VSL to VCL. Fresh and post-treated sperm samples were activated with a 0.30% NaCl solution, and the forward motility survival time was measured as the point at which movement completely ceased. Each group was analyzed three times.

Cryopreservation procedure. Diluted sperm samples were equilibrated at a temperature of 4.00 ˚C in an icebox for 10 min. For the freezing process, in an adjustable insulated box, the straws (0.50 mL) were placed on a horizontally oriented tray positioned 5.00 cm (around −180 ˚C) above the surface of liquid nitrogen for 10 min.[8] The straws were then immersed in liquid nitrogen and stored for 14 days. Straws were thawed in a 30.00 ˚ C water bath for 15 sec just before analyzing motility measurements after 14 days.[16,37]

Oxidative stress indices. Sperm samples were centrifuged at 3,000 *g* for 15 min at 4.00 ˚ C using an LD5-

2B centrifuge from Beijing Shiningsun Technology, Beijing, China. The resulting pellet was then homogenized with a glass-Teflon homogenizer in a 1.15% KCl at a 1 : 10 w/v ratio, and centrifuged at 3,500 rpm for 15 min. The levels of malondialdehyde (MDA; nmol g^{-1} cell) and reduced glutathione (GSH; µmol g^{-1} cell), as well as the enzymatic activities of superoxide dismutase (SOD; U mg^{-1} protein), catalase (CAT; k g^{-1} protein), and glutathione peroxidase (GPx; U g^{-1} protein) were evaluated in the sperm cells using a spectrophotometer (UV-1800; Shimadzu, Kyoto, Japan). To assess lipid peroxidation, thiobarbituric acid-reacting substances were measured according to the method outlined by Placer *et al*.[38] The SOD activity was assessed based on the methodology described by Sun *et al*.,[39] while CAT activity was measured according to Góth's method.[40] The activity of GPx was determined using cumene hydroperoxide and GSH as co-substrates, following the approach of Matkovics *et al*.[41] The GSH levels were determined according to the method described by Chavan *et al*.[42] The protein concentrations were measured according to the modified Lowry method.[43] The absorbance readings for thiobarbituric acid-reacting substances, GSH, SOD, CAT, and GPx were recorded at 532, 412, 560, 405, and 412 nm, respectively. All measurements were conducted in triplicate.

Statistical analysis. Data analysis was conducted using SPSS Software (version 27.0; IBM Corp., Armonk, USA) results presented as mean ± standard deviation. One-way analysis of variance was used to evaluate the data of different treatments. For multiple comparisons, the Duncan multiple comparison test was used. Principal component analysis and correlation analysis were carried out by PAST Software (version 4.03; Palaeontologia Electronica, Oslo, Norway) to investigate the relationships among the variables. A significance level of $p < 0.05$ was established for the analysis.

Results

In this study, data of three different treatments were presented. Motility rates and duration of fresh sperm for T1, T2, and T3 were 96.67 ± 2.89% and 52.67 ± 6.43 sec, 81.67 ± 1.28% and 50.33 ± 4.04 sec, and 90.33 ± 7.51% and 63.33 ± 1.75 sec, respectively. Table 1 summarized the motility parameters of sex-reversed rainbow trout sperm following three treatments. In T1, the highest sperm motility duration was in group with AA (1.00 mM) + L (1.00 mM) with a mean of 43.00 ± 4.76 sec, and the lowest motility duration was in control group with a mean of 30.50 ± 2.59 sec (*Fisher [F]* = 18.563 and $p = 0.000$). In T2, the highest sperm motility duration was in AA (1.00 mM) + C (2.00 mM) group with a mean of 49.00 ± 1.00 sec, and the lowest motility duration was in C (2.00 mM) group with a mean of 23.33 ± 0.58 sec ($F = 74.743$ and $p = 0.000$). In T3, the highest sperm motility duration was in AA (1.00 mM) + T (2.00 mM) group with a mean of 37.33 ± 2.52 sec, and the lowest motility duration was in T (2.00 mM) group with a mean of 20.00 ± 2.00 sec ($F = 16.351$ and $p = 0.001$).

Table 1. The motility parameters of sex-reversed rainbow trout (*Oncorhynchus mykiss*) sperm after thawing following three treatments, including incorporation of ascorbic acid (AA), and L-tryptophan (L), cysteine (C), and α-tocopherol (T) to the extender media.

Groups	Progressive motility (%)	Motility duration (sec)	Curve speed (µm sec^{-1})	Linear speed (µm sec^{-1})	Average path velocity (µm sec^{-1})	Linearity index	Straightness index
***Treatment* 1**							
Control	72.67 ± 2.50^{ab}	30.50 ± 2.59^{a}	86.82 ± 1.53^{a}	57.84 ± 1.37^{a}	33.33 ± 3.06^{a}	38.67 ± 1.55^{a}	47.30 ± 2.61^{a}
AA	74.00 ± 1.29^{ab}	32.80 ± 1.10^{a}	90.51 ± 6.03^{a}	55.37 ± 3.31^{a}	28.52 ± 1.44^{a}	35.47 ± 2.02^{a}	40.57 ± 1.22^{a}
L	65.00 ± 5.77^{a}	41.75 ± 3.95^{b}	97.68 ± 1.76^{a}	70.58 ± 2.92^{a}	45.72 ± 3.37^{a}	48.14 ± 1.07^{a}	52.26 ± 2.66^{a}
AA + L	78.14 ± 5.08^{b}	43.00 ± 4.76^{b}	109.35 ± 2.78^{a}	69.69 ± 2.62^{a}	48.80 ± 2.03^{a}	46.11 ± 1.57^{a}	47.78 ± 8.63^{a}
***F* value**	2.790	18.563	1.099	0.242	0.813	0.409	0.444
***p* value**	0.070	0.000	0.394	0.865	0.515	0.750	0.727
Treatment 2							
Control	70.00 ± 2.74^{a}	25.67 ± 1.15^{a}	71.07 ± 3.21^{a}	17.66 ± 3.68^{a}	34.36 ± 1.07^{a}	27.92 ± 1.19^{a}	52.99 ± 8.09^{a}
AA	95.00 ± 1.16^{b}	45.00 ± 5.00^{b}	61.41 ± 2.83^{a}	19.93 ± 8.02^{a}	35.13 ± 1.59^{a}	33.03 ± 2.12^{a}	57.69 ± 4.06^{ab}
C	81.67 ± 4.15^{c}	23.33 ± 0.58^{a}	57.72 ± 4.17^{a}	17.69 ± 1.31^{a}	30.63 ± 2.19^{a}	30.92 ± 2.97^{a}	57.25 ± 4.37^{ab}
AA + C	72.08 ± 9.12^{d}	49.00 ± 1.00^{b}	46.78 ± 2.23^{a}	20.34 ± 2.70^{a}	30.17 ± 1.75^{a}	43.66 ± 7.39^{a}	67.26 ± 5.56^{b}
***F* value**	39.707	74.743	0.337	0.095	0.090	3.270	3.296
***p* value**	0.000	0.000	0.799	0.961	0.963	0.080	0.079
***Treatment* 3**							
Control	77.47 ± 9.96^{a}	25.67 ± 1.15^{a}	36.82 ± 2.03^{a}	14.64 ± 1.41^{a}	21.55 ± 6.30^{a}	46.11 ± 1.80^{a}	70.44 ± 1.30^{ab}
AA	85.00 ± 1.25^{a}	35.33 ± 6.11^{b}	55.02 ± 1.68^{a}	25.97 ± 3.83^{a}	36.24 ± 7.08^{b}	48.75 ± 7.78^{a}	72.15 ± 4.07^{a}
T	61.91 ± 1.46^{ab}	20.00 ± 2.00^{a}	49.72 ± 1.71^{a}	13.70 ± 1.50^{b}	24.87 ± 3.75^{ab}	29.52 ± 8.57^{a}	55.53 ± 5.74^{b}
AA + T	68.22 ± 4.65^{c}	37.33 ± 2.52^{b}	47.95 ± 1.52^{a}	20.06 ± 3.61^{c}	31.20 ± 6.81^{ab}	43.42 ± 8.30^{a}	64.86 ± 6.59^{ab}
***F* value**	11.643	16.351	0.579	12.018	3.421	1.663	2.556
***p* value**	0.003	0.001	0.645	0.002	0.073	0.251	0.128

$^{a-d}$ Different letters indicate significant differences compared to the control in each column ($p < 0.05$).

The results indicated that there were no significant differences in sperm motion parameters (VCL, VAP, LIN, and STR) among the various treatments ($p > 0.05$).

The MDA levels in sperm were slightly increased in all treatments compared to the control group (Table 2). In T1, GPx and SOD activities decreased in all groups compared to the control group. In T2, GPx and SOD activities had the highest level in AA (1.00 mM) group compared to the control group. In T3, GPx and CAT activities increased in all groups compared to the control group.

Principal component analysis provides changes in sperm quality parameters and oxidative stress indices. In T1, CAT was strongly related to VSL, VAP, LIN, and STR. Motility rate had negative scores featuring sperm. In T2, motility duration was strongly related to MDA, GPx, LIN, and STR. Motility rate and SOD had negative scores featuring sperm. In T3, motility duration was strongly related to motility rate and VSL (Figs. 1 and 2).

Discussion

The present study gives the first overview of the improving impact of combinations of different anti-oxidants (AA-L, AA-, C, and AA-T) during long-term storage of sperm. The success of broodstock management depends pivotal on gamete quality. Understanding the effects of combination of different anti-oxidants in the extender media on gametes facilitates the optimization of cryopreservation protocols.[32] In addition, gamete quality affects the offspring and subsequent growth performance of larvae and juveniles.[44] This study provides the first comparative analysis of the effects of combinations of different anti-oxidants (AA-L, AA-C, and AA-T) on sperm of sex-reversed rainbow trout during cryopreservation, although research on sperm quality and optimization of cryopreservation protocol has primarily focused on sex-reversed female rainbow trout until now.[1,3,4,45-49]

Table 2. Malondialdehyde (MDA) and reduced glutathione (GSH) levels, and glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) activities of sex-reversed rainbow trout (*Oncorhynchus mykiss*) sperm following three treatments, including incorporation of ascorbic acid (AA), and L-tryptophan (L), cysteine (C), and α-tocopherol (T) to the extender media.

Groups	MDA (nmol g^{-1} cells)	GSH (µmol g^{-1} cells)	GPx (U mg^{-1} protein)	CAT (k g^{-1} protein)	SOD (U mg^{-1} protein)
Treatment 1					
Control	5.41 ± 0.73^{a}	0.15 ± 0.03^{a}	76.17 ± 2.75^{a}	48.12 ± 2.67^{a}	1.08 ± 0.34^{a}
AA	7.47 ± 1.24^{b}	0.19 ± 0.03^{b}	40.98 ± 1.76^{ab}	57.71 ± 1.67^{a}	0.37 ± 0.20^{b}
L	8.10 ± 1.18^{b}	0.13 ± 0.02^{a}	38.19 ± 2.33^{b}	58.49 ± 7.52^{a}	0.35 ± 0.08^{b}
AA + L	8.85 ± 0.96^{b}	0.20 ± 0.03^{b}	37.38 ± 1.62^{b}	54.89 ± 1.08^{a}	0.31 ± 0.08^{b}
***F* value**	10.723	6.012	3.069	0.178	14.282
***p* value**	0.001	0.010	0.069	0.908	0.000
***Treatment* 2**					
Control	9.70 ± 1.40^{a}	0.19 ± 0.07^{a}	28.54 ± 2.58^{a}	59.03 ± 7.50^{a}	0.48 ± 0.05^{ab}
AA	12.99 ± 1.14^{b}	0.20 ± 0.04^{a}	51.86 ± 1.29^{a}	50.19 ± 2.71^{a}	0.52 ± 0.07^{a}
C	13.24 ± 1.11^{b}	0.27 ± 0.07^{a}	34.49 ± 2.97^{a}	46.91 ± 7.81^{a}	0.46 ± 0.06^{ab}
AA + C	15.04 ± 3.00^{b}	0.24 ± 0.04^{a}	37.84 ± 2.54^{a}	91.47 ± 2.04^{b}	0.40 ± 0.09^{b}
***F* value**	6.012	1.893	0.653	3.874	2.287
***p* value**	0.008	0.177	0.599	0.038	0.123
Treatment 3					
Control	17.89 ±1 .93^{a}	0.20 ± 0.08^{a}	27.78 ± 7.47^{a}	26.84 ± 5.67^{a}	0.34 ± 0.10^{a}
AA	17.62 ± 1.21^{a}	0.27 ± 0.13^{a}	34.42 ± 7.99^{a}	37.11 ± 2.18^{a}	0.25 ± 0.16^{a}
T	19.58 ± 1.70^{a}	0.27 ± 0.23^{a}	42.25 ± 4.30^{a}	43.25 ± 6.71^{b}	0.22 ± 0.23^{a}
AA + T	19.43 ± 1.20^{a}	0.22 ± 0.02^{a}	77.56 ± 2.13^{b}	131.70 ± 1.44^{c}	0.30 ± 0.12^{a}
***F* value**	0.904	0.593	8.768	17.476	0.525
***p* value**	0.473	0.633	0.005	0.000	0.675

$^{a\text{-}c}$ Different letters indicate significant differences compared to the control ($p < 0.05$).

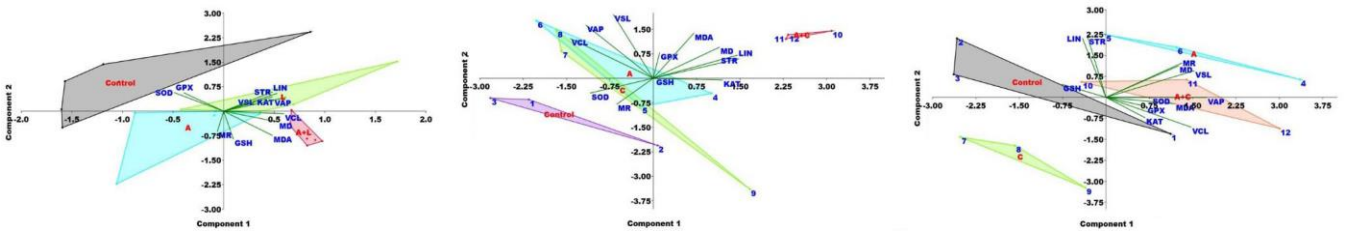

Fig. 1. Biplot of principal component analysis of variables (motility rate [MR], motility duration [MD], curvilinear velocity [VCL], straight-line velocity [VSL], velocity of the average path [VAP], straightness [STR], linearity of movement [LIN], malondialdehyde [MDA], reduced glutathione [GSH], glutathione peroxidase [GPx], superoxide dismutase [SOD], and catalase [CAT]) in incorporation of ascorbic acid (AA), and L-tryptophan (L), cysteine (C), and α-tocopherol (T) to the extender media of sex-reversed rainbow trout sperm.

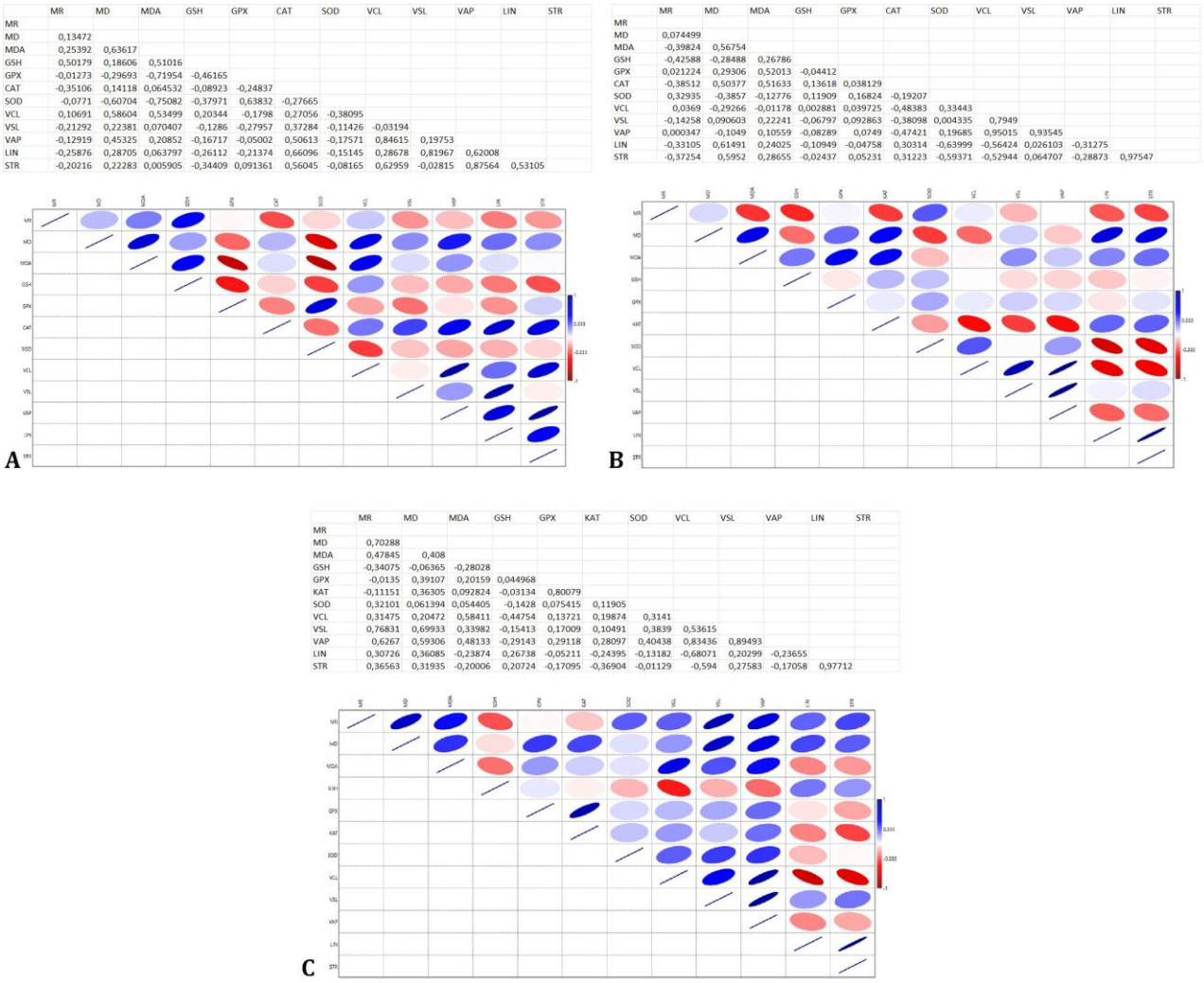

Fig. 2. Correlation analysis of variables in incorporation of ascorbic acid and **A)** L-tryptophan, **B)** cysteine and **C)** alpha-tocopherol to the extender media. MR: Motility rate; MD: Motility duration; VCL: Curvilinear velocity; VSL: Straight-line velocity; VAP: Velocity of the average path; STR: Straightness; LIN: Linearity of movement; MDA: Malondialdehyde; GSH: Reduced glutathione; GPx: Glutathione peroxidase; SOD: Superoxide dismutase; CAT: Catalase.

Additive molecules with anti-oxidant properties provide sperm cells protection against damage caused by ROS and mitigate oxidative injury.[15,17,45-47] Key anti-oxidant enzymes, including SOD, GPx, and CAT, play a crucial role in defending against oxidative stress.[50] In the current study, MDA levels increased in all treatments compared to the control group. The increased vulnerability of membrane phospholipids and the high concentration of polyunsaturated fatty acids in spermatozoa might be linked to increased lipid peroxidation and oxidative stress during cryopreservation process.[11,17,50] In T1, GPx and SOD activities decreased in all groups compared to the control group. In T2, GPx and SOD activities had the highest level in AA (1.00 mM) group compared to the control group. The increment in SOD levels may be associated with the defense against oxidative stress due to the elevated MDA levels.[50] In T3, GPx and CAT activities increased in all groups compared to the control group. The elevation in CAT and GPx activities can be attributed to their insufficiency in preventing oxidative stress.[13,17] The concentrations of anti-oxidants are inadequate for reducing ROS levels and safeguarding membrane structure and cell function. Additionally, the frozen- thawed process can disrupt Ca^{2+} homeostasis, a key factor in cellular processes, potentially leading to increased ROS production and mitochondrial dysfunction.[50] Furthermore, as previously reported that in the presence of transition metals, AA may increase the reactivity of radicals, making them more destructive and leading to the production of additional free radicals.[32]

Regarding the motility duration and sperm movement parameters analyzed in the current study, our findings align with previous reports that anti-oxidants in extender media have a beneficial effect on improving sperm quality. Interestingly, our results showed that motility durations enhanced at combinations of different anti-oxidants (AA

[1.00 mM] + L [1.00 mM], AA [1.00 mM] + C [2.00 mM], and AA [1.00 mM] + T [2.00 mM]), while AA caused an increase in motility rate at all treatments compared to the control group. In agreement with our data, Kocabaş *et al*.[32] obtained the best results for motility rate and duration from the combination of L (1.00 mM)/AA (1.00 mM) during short-term sperm storage of rainbow trout (*O. mykiss).* Martínez-Páramo *et al*.[20] stated that freezing media supplemented with T and AA resulted in a notable improvement in total motility compared to the control extender in European sea bass (*D. labrax*). In contrast with results of motility rate and duration, in the present work, sperm movement parameters (VCL, VSL, VAP, LIN, and STR) fluctuated in all treatments in consistent with findings by Cabrita *et al*.,[31] demonstrated that the supplementation of anti-oxidants (vitamins and amino acids) to extender did not cause a significant improvement in motility parameters, including TM (total motility), PM (progressive motility), VCL, VSL, or LIN in sperm cryopreservation of the gilthead seabream.

Principal component analysis provides an evaluation of the relationships between the variables. In T1, CAT showed a strong association with VSL, VAP, LIN, and STR, while motility rate had negative scores characterizing sperm. In T2, motility duration was strongly correlated with MDA, GPx, LIN, and STR, and both motility rate and SOD exhibited negative scores characterizing sperm. In T3, motility duration had a strong relationship with motility rate and VSL. The noted difference in correlation among variables can be explained with anti-oxidant type and combined impact.

In conclusion, the optimal combination of anti-oxidants (AA [1.00 mM] + L [1.00 mM]) at the tested concentrations caused prolonging effect in sperm motility in post-thaw samples. Species-specification, type, concentrations, and combination of anti-oxidants affected anti-oxidant action during sperm cryopreservation. To prevent further cryodamage, additional research is needed to assess the optimal concentration or combination of anti-oxidants, particularly AA and L.

Acknowledgments

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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