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Dietary antioxidants supplementation increases sperm motility, fertility and oxidative enzyme activity during cryopreservation of koi carp *Cyprinus carpio* Linnaeus, 1758

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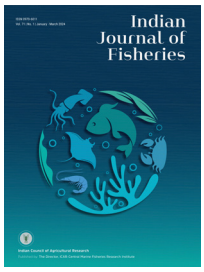

Abstract

In aquaculture, oxidative stress is considered a major problem as it impairs sperm motility and cell viability, especially during cryopreservation. This can be overcome by dietary supplementation of antioxidants, which is essential during gametogenesis and spermiation. Hence, the present study aimed at evaluating the influence of dietary antioxidants on sperm quality and oxidative enzyme activity during cryopreservation. Experimental fishes (*Cyprinus carpio* L.) were fed with feed supplemented with vitamin C and E at three different concentrations, which consisted of 6 treatments and a control (without vitamin inclusion). The experimental feed was fed to the fish until satiation for 60 days, after which, milt was collected from the fish and cryopreserved for 30 days. After 30 days of cryopreservation, fertilisation was done using cryopreserved milt and fresh milt. The oxidative enzyme activities, such as glutathione peroxidase, catalase and superoxide dismutase, were recorded. The values of motility duration, fertilisation and hatching rate showed an overall linear and quadratic significant differences ($p<0.05$) among the treatments. The histological analysis of the ovary was also documented to analyse the effect of antioxidants on the ovarian development. Feed supplemented with vitamin E at 200 mg kg^{-1} (T4) exhibited higher motility value, fertilisation and hatching rates and oxidative enzyme activities in comparison to other treatments.

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Introduction

World aquaculture production and the human population are both showing increasing trends. Enhancing the reproductive performance of fish is the only way to increase production from aquaculture to meet the demand. However, Félix *et al*. (2021) reported that a higher amount of polyunsaturated fatty acids in fish spermatozoal membranes, makes them susceptible to oxidative stress, thereby adversely affecting reproductive performance. They also mentioned that this could be overcome by appropriate dietary antioxidant supplementation in broodstock, enabling the transfer of beneficial compounds to the offspring (Felix *et al*., 2021). Oxidative stress can impair sperm motility, cell viability and functionality, and DNA integrity (Cabrita *et al*., 2014). Seminal plasma serves as a natural antioxidant medium that helps protect spermatozoa from oxidative damage (Aitken and Drevet, 2020). In aquaculture, stressors such as high stocking densities, temperature fluctuations, frequent handling and prophylactic treatments can enhance oxidative stress in fish. Therefore, diets enriched with antioxidants play a pivotal role in balancing the stress caused by these factors particularly during gametogenesis and spermiation. Several studies have highlighted the importance of antioxidants in protecting fish sperm from oxidative damage (Mansour *et al*., 2006; Martínez-Páramo *et al*., 2013; Cabrita *et al*., 2014; Wischhusen *et al*., 2019).

Vitamin C (ascorbic acid) and E (α-tocopherol) are the most important non-enzymatic

antioxidants present in seminal plasma and play significant roles in fish reproduction (Izquierdo *et al*., 2001). Vitamin C, a water-soluble antioxidant, is an essential dietary nutrient for fish (Dabrowski and Ciereszko, 2001). It is a highly effective antioxidant and serves as a cofactor for several enzymatic reactions, including collagen hydroxylation and carnitine synthesis, thereby supporting normal physiological functions (Okhionkpamwonyi and Edema, 2017). In addition, vitamin C plays a major role in bone development and overall growth in fish (Gbadamosi *et al*., 2013). Vitamin E, a fat-soluble antioxidant, also plays a vital role in the reproductive physiology of fish (Watanabe, 1985). It protects polyunsaturated fatty acids (PUFAs) and cholesterol in the cellular and subcellular membranes from lipid peroxidation (Roy and Mollah, 2009) and improves the activity of other antioxidant systems (Ahmadi *et al*., 2016). Vitamin E deficiency has been associated with impaired reproductive performance, including delayed ovarian development in carp (Watanabe and Takashima, 1977).

Cryopreservation is an important biotechnological tool for the production of high quality seeds and the production of genetically improved fish stocks. However, the cryopreservation process can alter the antioxidant components of the spermatozoa, by altering the activity of important antioxidant enzymes including, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) (Lahnsteiner *et al*., 2010; Lahnsteiner *et al*., 2011). The cooling and thawing rates are regarded as the most critical phases during cryopreservation, as they can induce oxidative stress and compromise sperm quality (Franks, 1985). Therefore, the present study was designed to investigate the effect of antioxidants dietary supplementation with vitamins C and E at different concentrations on spermatological properties, oxidative enzyme activities and cryopreservation success in koi carp (*Cyprinus carpio* L.).

Materials and methods

The experiment was conducted following the procedures of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests (Animal Welfare Division), Government of India, on care and use of animals in scientific research.

Experimental fish and feeding trial

The brooders (300 nos.) of adult koi carp (*C. carpio*) were procured and maintained in cement tanks of 500 l capacity. Each experimental tank was stocked with 10 fish in triplicate. The average body weight of female fish was 54±0.5 g, and that of male fish was 51±0.5 g. The experimental feeds were prepared according to the feed formulation as per Betsy *et al.* (2021). The experiment was conducted following a completely randomized design (CRD) with seven treatments, including a control, each in triplicate. Vitamin C and E were added to the feed at 3 different concentrations, *viz*., 200, 400 and 600 mg kg^{-1} feed. The details of feed formulation and proximate composition of the feed are given in Table 1. The experimental feed was fed to the fish until satiation for 60 days.

All measured parameters were averaged for each tank, and the resulting means (n=3 per treatment) were used for statistical analysis to avoid pseudo-replication.

Once a week, a 50% water exchange was done, and the excess feed and faecal matter were removed daily by siphoning to maintain the water quality. The water quality parameters were monitored daily following standard methods (APHA, 2005). Throughout the experimental period, all parameters remained within the optimal range for koi carp culture, with mean values of 28±1°C for water temperature observed at, 7.6±0.2 for pH, 5.5 - 6.1±0.3 mg l^{-1} for dissolved oxygen (DO) , 0.02±0.01 mg l^{-1} for ammonia-N, 0.06±0.01 mg l^{-1} for nitrite-N, 10±0.01 mg l^{-1} for nitrate-N, 564±12 mg l^{-1} for total hardness and 166.7±11 mg l^{-1} (as $CaCO_3$) for total alkalinity.

Milt collection

Milt was collected from male fish exhibiting oozing milt upon gentle abdominal pressure in each treatment tank at 15-day intervals to

Table 1. Feed formulation and proximate composition of the experimental diets (%)

	Diet (% of inclusion)						
Ingredients	C	T1	T2	T3	T4	T5	T6
Corn flour	13	13	13	13	13	13	13
Soybean meal	35	35	35	35	35	35	35
Groundut oil cake (GNOC)	20	20	20	20	20	20	20
Rice bran	6	6	6	6	6	6	6
Fish meal	5	5	5	5	5	5	5
Cassava	5.5	5.48	5.46	5.44	5.48	5.46	5.44
Wheat flour	15	15	15	15	15	15	15
Vitamin E	0	0	0	0	0.02	0.04	0.06
Vitamin C	0	0.02	0.04	0.06	0	0	0
Mineral premix[a]	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Proximate composition of feed (%)							
Crude protein	32.37	32.29	32.25	32.62	32.50	32.12	32.06
Crude lipid	1.93	2.62	2.03	2.08	2.08	2.24	2.27
Moisture	8.84	8.01	8.82	8.04	8.34	8.36	8.15
Ash	11.3	10.73	11.61	11.49	11.48	11.25	11.32

[a] Composition of mineral premix (quantity per kg): Magnesium – 2,800 mg, Iodine – 7.4 mg, Iron – 7,400 mg, Copper – 1,200 mg, Manganese – 11,600 mg, Zinc – 9,800 mg, Chlorides Cobalt – 4 mg, Potassium – 100 mg, Selenium – 4 mg, Calcium Carbonate – 27.25%, Phosphorous – 7.45 mg, Sulphur – 0.7 mg, Sodium – 6 mg, Calpan – 200 mg, Aluminium – 1,500 mg and Choline chloride – 10,000 mg

assess the spermatological parameters. Milt samples Milt collection was done by gentle stripping as described by Kurokura *et al*. (1984) and Lubzens *et al*. (1997), and the milt samples were collected aseptically into a sterile, pre-labelled vials, ensuring absence of contamination (Betsy *et al*., 2019).

Cryopreservation of milt

After 60 days of feeding the fish with experimental feeds, milt was collected from 5 male fish each per treatment groups for cryopreservation. The collected milt was diluted with 0.85% physiological saline solution as extender and 10% dimethyl sulfoxide (DMSO) as cryoprotectant at a 1:40 dilution ratio (Betsy *et al*., 2019). No equilibration time was given after dilution as suggested by Betsy *et al*. (2019). The diluted milt was loaded into straws of 0.25 ml volume and was rapidly frozen using a programmable controlled rate freezer (PLANER, Kryo 560-16). A three-step protocol was followed for sperm cryopreservation. The samples were first cooled from 5 to -4°C at a rate of 2°C min^{-1} (Ramp 1 min), and then cooled from -4 to -80°C (@ 5°C min^{-1}), followed by direct transfer to liquid nitrogen (LN_2). Upon completion of the programmed freezing curve and attainment of -80°C, the straws were removed from the programmable freezer and transferred to a BA11 Cryocan (IBP, India) for storage at -196°C in canisters fitted with goblets. The cryocan was maintained with LN_2 at a depth of 20 to 23 cm from the bottom, and the LN_2 level was monitored weekly using a dipstick.

Five straws from each treatment were taken out from the cryocan and thawed at 30˚C for 30 s in a serological water bath (Bozkurt *et al*., 2005). The rapidly thawed straws were wiped using tissue paper to remove the water drops, and the thawed milt from the straws were drained properly into sterile vials for subsequent analyses. Sperm motility and fertilisation ability were assessed after 30 days of cryopreservation. Oxidative enzyme activities such as glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) were analysed to assess the oxidative status of the cryopreserved sperm.

Histological analysis of ovary

The ovaries of the fish sampled from each treatment were dissected for histological analysis once every fortnight. Two fish from each treatment were frozen to death (Huang *et al*., 2020) for dissecting the gonad. The samples from the ovary were collected from anterior, medial and posterior portions and stored in Bouin's fluid for 24 h, processed, embedded in paraffin and sectioned into 5 to 6 µm-thick sections with the help of a microtome (Shirali *et al*., 2011). These sections were kept in an oven at 40°C overnight and were stained with hematoxylin-eosin (Cho *et al*., 2014; Balci and Aktop, 2019) and observed under a compound microscope (NIKON E360). Histological observations observations and interpretations were carried out following Kirschbaum and Formicki (2019).

Assessment of sperm motility, oxidative enzyme activity and fertilisation ability

Motility assessment

The milt samples were examined under a phase contrast microscope (NIKON E360) at x200 magnification to assess motility duration. For the analysis, 10 µl of milt sample was activated with 100 µl of tap water on a clean glass slide and sperm movement was observed under the microscope (Secer *et al*., 2004; Betsy *et al*., 2017). Sperm motility was assessed both before and after dilution of the milt samples.

Oxidative enzyme activity

To assess the antioxidant enzyme activity, the cryopreserved samples were centrifuged at 5000 g for 5 min at 4°C. The supernatant was discarded, and the sperm pellets were stored at −80°C for further processing. The cell extracts were prepared according to Lahnsteiner *et al*. (1998), and stored at −80°C for evaluation of enzyme activity.

Glutathione peroxidase

Glutathione peroxidase (GPx) activity was determined following Figueroa *et al*. (2018) using an indirect quantitative colourimetric assay kit (BioVision®). The GPx activity was measured by monitoring the change in absorbance at 340 nm for 10 min at 25°C using a multimode microplate reader (SynergyTM HT). The enzyme activity was calculated from a standard NADPH curve and expressed as nmol min^{-1} ml^{-1}.

Catalase

Catalase activity was estimated following Figueroa *et al*. (2018) using OxiSelect™ catalase activity assay kit (Cell Biolabs, INC®). Enzyme activity was quantified by measuring the absorbance at 520 nm using a multimode microplate reader (SynergyTM HT), Catalase activity was calculated from a standard catalase calibration curve using a second-order polynomial equation and expressed as U ml^{-1}.

Superoxide dismutase

The activity of superoxide dismutase (SOD) was estimated following the protocol by Martınez-Páramo *et al*. (2012). The absorbance was measured at 505 nm and SOD activity was expressed as units of enzyme per mg of protein. Protein content was estimated according to Lowry *et al*. (1951) using the Bio-Rad DC protein assay kit (Bio-Rad, Germany).

Artificial fertilisation

The dry method of *in vitro* fertilisation was employed following Sultana *et al*. (2009) and Aliniya *et al*. (2013). Fresh milt was collected from the broodfish and diluted with the same extender and dilution ratio used for cryopreserved sperm to minimise experimental variation. Eggs that were fertilised with fresh milt served as control. Cryopreserved spermatozoa were thawed for 30 s and immediately added over the eggs and gently mixed with a feather. After proper mixing, water was added to the milt-egg mixture and mixed for one minute (Sultana *et al*., 2009). Subsequently, the fertilised eggs were transferred to nylon filaments and incubated in hapas. Fertilisation rate and hatching rate were determined as per Bromage and Cumaranatunga (1988) and Hanjavanit (2008), respectively.

Statistical analysis

Data on sperm motility, fertilisation rate, hatching rate, and oxidative enzyme activities were analysed using the General Linear Model (GLM) procedure in SPSS version 22 (SPSS Inc., USA). Differences among treatment means were tested using one-way analysis of variance (ANOVA), and significance was determined at $p<0.05$. Duncan's multiple range test was used for *post hoc* comparison of means. Polynomial contrast analysis (linear and quadratic) was applied independently within each antioxidant group (vitamin C and E) across various inclusion levels (200, 400, and 600 mg kg^{-1}) to evaluate dose-dependent trends.

Results and discussion

Effect of antioxidants on the ovary

In the present study, the ovarian histology was included to provide complementary insights into the effects of dietary antioxidants on overall reproductive physiology, particularly gonadal development and maturation. As it can be seen from Table 2 and Fig. 1, the histological analysis of the ovary of koi carp at various sampling points revealed the presence of ova at different stages of development, like yolk vesicle stage (YVS), peri-nucleolus stage (PNS), primary vitellogenesis stage (PVS) and secondary vitellogenesis stage (SVS). The posterior ovary had ova at the maturing stage compared to ova analysed from the anterior and middle portions in almost all the treatments. Fishes reared under the control group showed SVS only on the 60th day in the posterior section of the ovary. Among vitamin C, fish fed with 600 mg kg^{-1} incorporated feed exhibited a matured ovary from the 30th day onwards.

When fish were fed with vitamin E, the matured ovary was noticed from the 15th day onwards, irrespective of the vitamin concentration. This is in accordance with Watanabe (1985), who mentioned that deficiency of vitamin E retards the gonadal development in common carp. In all the samples, the posterior portion of the ovary had a higher number of SVS and few YVS, PNS and CNS, which

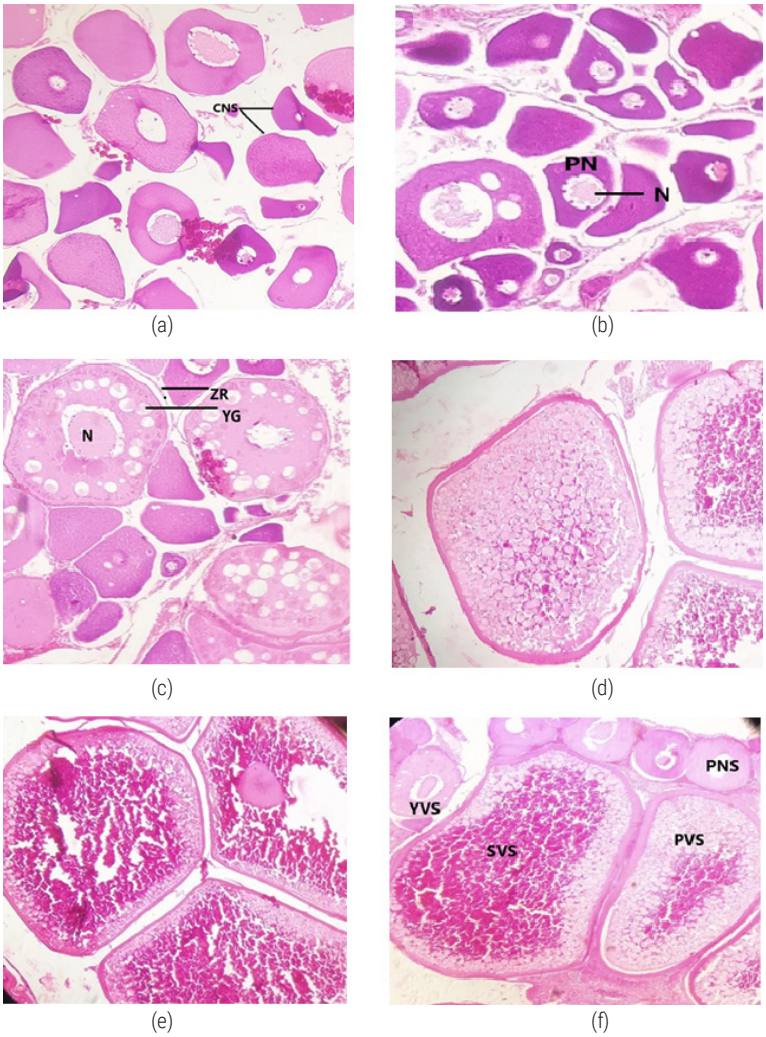

Fig. 1. Photomicrographs of histological sections of the ovary after dietary antioxidants supplementation in koi carp brooders. (a) Chromatin nucleolus stage; (b) Perinucleolus stage; (c) Yolk vesicle stage; (d) Primary vitellogenesis stage; (e) Secondary vitellogenesis stage; (f) different oocyte developmental phases. N- Nucleus; YG- Yolk globule; ZR- Zona radiata

Table 2. Histological observations in the ovary of koi carp fed with vitamin C and E at different concentrations

Treatment	Portion of ovary	15th day of sampling	30th day of sampling	45th day of sampling	60th day of sampling
C	A	PNS	CNS, PNS, YVS	CNS, PNS, YVS, PVS	CNS, YVS, PNS
	M	PNS, CNS	CNS, PNS, PVS	CNS, PNS, YVS, PVS	YVS, PNS, PVS, OT
	P	PVS	CNS, PNS, PVS	PNS, PVS	YVS, SVS
T1	A	PNS, YVS	SVS, CNS, PNS	OT, YVS, PVS	Very few PNS, few PVS
	M	YVS	PVS, SVS	YVS, SVS	Few PNS, OT, PVS
	P	YVS, few CNS	PVS, SVS	SVS	YVS, PVS
T2	A	PNS, CNS	CNS, PNS, YVS, OT	YVS, PVS, SVS	More PNS, YVS, OT
	M	PNS, CNS	CNS, PNS, YVS, OT	Few CNS and PNS, more YVS and SVS	More PNS, YVS, OT, PVS
	P	PVS, PNS, few CNS	PNS, YVS, PVS, OT	More YVS, very few SVS	More PNS, YVS, PVS
T3	A	Very few PNS	CNS, PNS, PVS	CNS, YVS, SVS	More PNS, few YVS and PVS
	M	PVS, PNS	PVS, SVS, YVS	CNS, YVS, SVS	More PNS, few YVS and PVS
	P	PNS, YVS	YVS, SVS	YVS, SVS	Very few PNS, more YVS, PVS and SVS
T4	A	PVS, PNS	PNS, PVS	YVS, SVS	More YVS and PVS, very few PNS
	M	CNS, PNS, YVS, OT	PNS, YVS, PVS	YVS, SVS	More CNS, YVS, few PVS, SVS
	P	CNS, PNS, YVS, SVS	PNS, YVS, PVS, SVS	SVS	More YVS, PVS and SVS
T5	A	Early PVS, PNS, SVS	YVS, SVS	CNS, PNS, SVS, OT	More CNS, PNS
	M	YVS, SVS	PVS, PNS, YVS, SVS	PVS, SVS	Few PNS, YVS and more PVS
	P	SVS	PNS, YVS, SVS	Very few YVS, lot of SVS	Few PNS, more PVS and SVS
T6	A	Few PNS, SVS	CNS, PNS, YVS, PVS, SVS	CNS, PNS, PVS, SVS	More PNS, SVS
	M	SVS	YVS, PNS, SVS	PNS, SVS, OT	PNS, SVS
	P	SVS	YVS, PNS, SVS	PNS, PVS, SVS	SVS

CNS - Chromatin nuclear stage; PNS - Peri nucleolus stage; YVS - Yolk vesicle stage; PVS - Primary vitellogenesis stage; SVS - Secondary vitellogenesis stage; OT- Ovarian tissue; A – Anterior ovary, M – Middle ovary and P- Posterior portion of the ovary.

indicated the presence of asynchronous ovaries since different oocyte developmental phases were seen together (Erkmen and Kirankaya, 2016). Smith and Walker (2004) stated that fish species with asynchronous ovaries have a prolonged breeding season, supporting that common carp is a multiple spawner. The results of the present study were in accordance with Shirali *et al*. (2011), who stated that common carp possess asynchronous ovaries and follicles of all sizes were seen at any time, but the stage of the ovary cycle was determined by the percentage of different types of follicles.

Effect of antioxidants on the cryopreserved milt

Sperm motility

The effects of antioxidants on the motility duration, fertilisation and hatching rate are presented in Table 3. The fresh milt collected from fish fed with vitamin E at 200 mg kg^{-1} feed exhibited the highest mean motility duration of 64.23 s and was followed by vitamin E at 600 mg kg^{-1} feed (61.32 s). After cryopreservation, the same group of milt exhibited significantly higher mean motility duration when compared to other treatments ($p<0.05$).

In raw milt, although the motility of fish in the control group was higher than that of fish fed with vitamin C at different concentrations and vitamin E at 400 mg kg^{-1} feed, after cryopreservation, the milt collected from fish reared in the control group exhibited lowest motility values. In raw milt, the lowest motility duration of 51.66 s was observed in milt collected from fishes fed with vitamin C at 600 mg kg^{-1} feed. However, after cryopreservation, the lowest motility duration of 27.26 s was recorded in the control group.

Oxidative stress is widely recognised as one of the main reason for sperm malfunctions during cryopreservation (Figueroa *et al*., 2018). Reactive oxygen species (ROS) targets fish spermatozoa

Table 3. Effect of antioxidants on the mean motility duration (s), fertilisation rate (%) and hatching rate (%) of cryopreserved koi carp spermatozoa

	Motility duration (s)		Fertilisation rate (%)		Hatching rate (%)	
	Fresh milt	Cryopreserved milt	Fresh milt	Cryopreserved milt	Fresh milt	Cryopreserved milt
T1	51.97^{ef}	40.59^{c}	89.2^{c}	63.33^{e}	76.17^{c}	41.41^{d}
T2	54.01^{d}	37.52^{e}	85.28^{d}	74.37^{c}	71.36^{d}	37.56^{f}
T3	51.66^{f}	38.22^{d}	79.36^{f}	62.39^{f}	62.58^{f}	41.53^{d}
T4	64.23^{a}	48.55^{a}	95.28^{a}	82.41^{a}	86.36^{a}	57.53^{a}
T5	52.41^{e}	36.38^{f}	81.81^{e}	68.36^{d}	67.29^{e}	43.39^{c}
T6	61.32^{b}	44.63^{b}	92.22^{b}	80.37^{b}	82.59^{b}	55.31^{b}
C	54.61^{c}	33.01^{g}	74.12^{g}	58.37^{g}	56.58^{g}	39.63^{e}
PSE	1.034	1.089	1.547	1.929	2.211	1.636

Data expressed as Mean (n=10, r=3); Mean values in the same column with different superscripts differ significantly ($p<0.05$). One way ANOVA was used following Duncan's multiple range test in SPSS-22.0. Estimated Marginal Means. PSE - Pooled Standard Error of the mean.

membrane, inducing lipid and protein peroxidation, damaging the midpiece as well as axoneme, causing DNA fragmentation and mitochondrial impairment (Lahnsteiner *et al*., 2010; Cabrita, *et al*., 2014; Beirão *et al*., 2015). These alterations lead to decreased sperm motility and fertilisation efficiency (Figueroa *et al*., 2018). Fish spermatozoa are particularly susceptible to cold shock and the osmotic and oxidative stress associated with freezing and thawing (Figueroa *et al*., 2018), making them highly vulnerable to cryoinjury. The findings of the present study are consistent with these observations, as sperm from the control group exhibited significantly reduced post-thaw motility after cryopreservation, indicating increased oxidative damage induced during the cryopreservation process.

According to Valcarce and Robles (2018), a certain amount of ROS is required for the normal functioning of sperm. In fish, seminal plasma has a specific defence system that can scavenge the excess ROS and prevent cellular damage (Kefer *et al*., 2009). Hence, antioxidants are necessary to overcome the adverse effects of excess ROS. In the present study, fish fed with a diet containing vitamin C and E at different concentrations had better motility values than the control group after cryopreservation. This indicates the effectiveness of antioxidant supplements in protecting the spermatozoa viability during cryopreservation (Martínez-Páramo *et al*., 2013; Figueroa *et al*., 2018). Martínez-Páramo *et al*. (2013) reported that the percentage of motile spermatozoa in seabass decreased significantly after cryopreservation, from 50.4±2.2% in fresh sperm to 20.7±3.3% in cryopreserved sperm without antioxidant additives. However, the addition of α-tocopherol and ascorbic acid to the freezing media significantly improved total motility (31.2±3.0% and 30.6±3.9%, respectively) in comparison with the extender control (20.7±3.3%).

Vitamin C and E can scavenge oxygen radicals in the aqueous phase and within the membrane, respectively (Niki, 1987). Among vitamin C and E, fish fed with vitamin E at 200 and 600 mg kg^{-1} feed gave the highest motility values. This is in accordance with Askari *et al*. (1994) who reported that vitamin E was more efficacious than vitamin C in improving post-thaw motility of human spermatozoa since vitamin E can inhibit lipid peroxidation reaction in the membrane by eliminating peroxyl (ROO–), alkoxyl (RO–), and other lipid-derived radicals (Halliwell and Gutteridge, 1999).

Fertilisation and hatching rate

The highest mean fertilisation rate of 82.41% was obtained in fishes fed with vitamin E at 200 mg kg^{-1} feed, which was followed by a mean fertilisation rate of 80.37% from fishes fed with vitamin E at 600 mg kg^{-1} feed ($p<0.05$). The lowest mean fertilisation rate of 58.37% was recorded from the control group (Table 3). Similarly, the hatching rate also coincided with the fertilisation rate. The highest mean hatching rate of 57.53% was obtained in fishes fed with vitamin E at 200 mg kg^{-1} feed, followed by the mean hatching rate of 55.31% from fishes fed with vitamin E at 600 mg kg^{-1} feed (Table 3).

Polynomial contrast analysis performed separately within each antioxidant group revealed that vitamin C treatments exhibited weak and inconsistent dose–response patterns, with no clear linear trend and only marginal quadratic effects. In contrast, vitamin E treatments showed a significant ($p<0.05$) quadratic response for motility duration, fertilisation rate, and hatching rate in both fresh and cryopreserved milt, with peak performance observed at 200 mg kg^{-1} (T4), followed by a decline at higher inclusion levels.

In the present study, the lowest mean fertilisation and hatching rate was obtained in control group, irrespective of the freezing protocol employed. This could be attributed to the exposure of cells to thermal shock and fluctuations in the oxygen availability during the freezing and thawing processes, which promotes the generation of ROS (Chatterjee *et al*., 2001; Li *et al*., 2010). The resulting oxidative stress enhances lipid peroxidation of the sperm plasma membrane, leading to structural damage and impaired sperm function. The decline in antioxidant capacity during cryopreservation may result from excessive ROS generation, with low endogenous antioxidant capacity to control lipid peroxidation and plasma membrane disruption. The decline in antioxidant capacity during cryopreservation may result from excessive ROS generation, with surpasses the endogenous antioxidant defense systems and promotes lipid peroxidation and plasma membrane disruption (Chen *et al*., 2010; Lahnsteiner *et al*., 2011; Liu *et al*., 2015). This suggests that the molecules remain sufficiently mobile at low temperatures, allowing cell ageing process. Protection against such oxidative damage largely depends on antioxidants and enzymes present in the seminal plasma and spermatozoa, which function to neutralise ROS and prevent oxidative damage (Li *et al*., 2010; Shaliutina-Kolešová *et al*., 2013; Dzyuba *et al*., 2014; Figueroa *et al*., 2018). Hence, fish fed with antioxidant supplemented diet exhibited improved reproductive performance and enhanced resilience of spermatozoa to cryopreservation induced oxidative stress.

Figueroa *et al*. (2018), during the cryopreservation of Atlantic salmon (*Salmo salar*) sperm, added vitamin C and E to the freezing medium, reducing superoxide production and lipid peroxidation and increasing spermatozoa membrane potential and motility and higher fertilisation capacity. According to Felix *et al*. (2021), most studies have focused on the supplementation of vitamins to the extender media, and they suggested that a long-term study is needed to conclude the effect of dietary antioxidant supplementation in enhancing fish sperm quality. In this line, Martínez-Páramo *et al*. (2013) evaluated the efficacy of antioxidant supplementation through diet and extender in European seabass as well as gilthead seabream and reported that dietary supplementation enhanced the antioxidant system, and together with extender supplementation, improved sperm quality after cryopreservation.

Oxidative enzyme activity

It was observed that GPx activity was significantly higher (13.62±0.02 nmol min^{-1} ml^{-1}) in milt samples collected from fishes fed with vitamin E at 600 mg kg^{-1} feed and cryopreserved while, the lowest value was recorded in control group (7.18±0.8 nmol min^{-1} ml^{-1}) (Fig. 2a). Similar trends were noticed for CAT (Fig. 2b) and SOD activities (Fig. 2c). The present results were in accordance with Figueroa *et al*. (2018). Shaliutina *et al*. (2013) and Dzyuba *et al*. (2014) stated that the protection of fish sperm directly depends on antioxidants and oxidative enzymes present in the seminal plasma, preventing the sperm from oxidative damage. Figueroa *et al*. (2018) reported lower CAT activity in Atlantic salmon sperm cryopreserved in media without antioxidant supplementation, which could be due to a lower reduction rate of $O_2^{\bullet-}$ to H_2O_2 by SOD activity, thus increasing levels of intracellular $O_2^{\bullet-}$ that inhibits CAT activity (Lahnsteiner *et al*., 2011). This might be the reason for low enzyme activity in the control group.

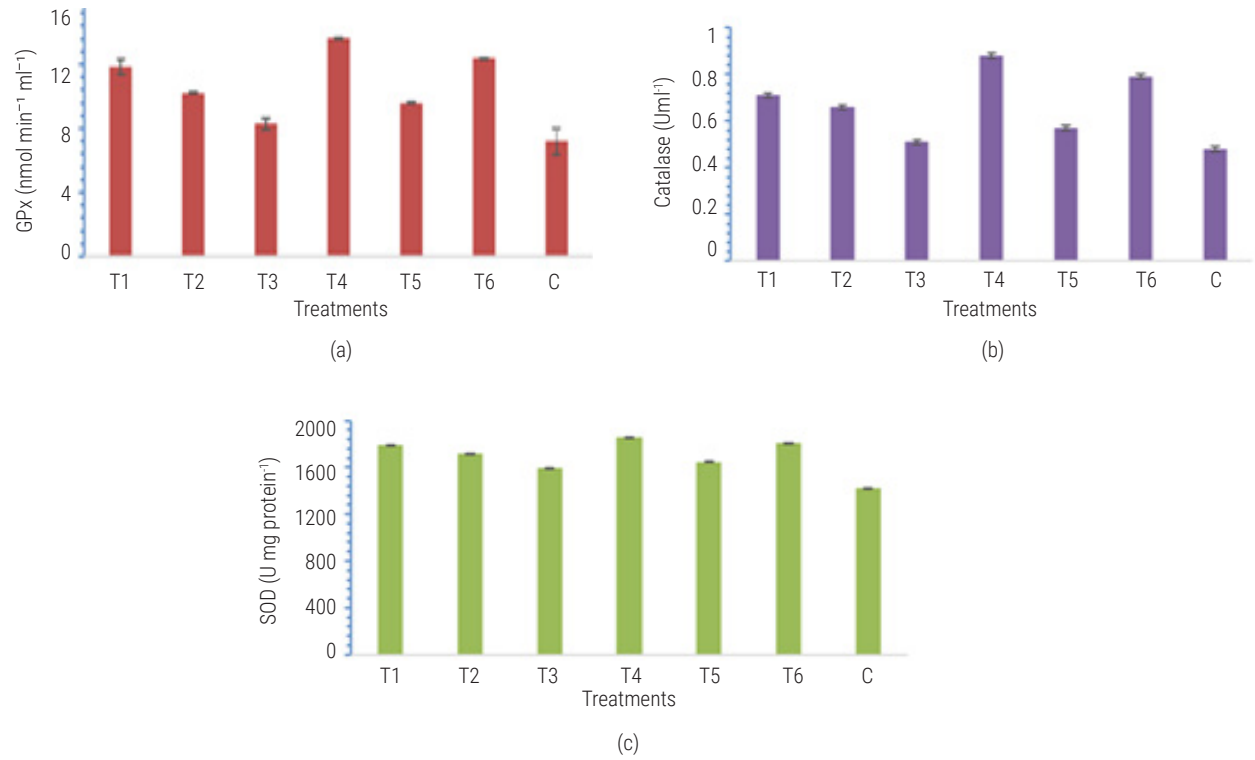

Fig. 2. Oxidative enzyme activity in the antioxidants incorporated diet of koi carp. (a) Glutathione peroxidase (nmol min^{-1} ml^{-1}); (b) Catalase (Uml^{-1}) and (c) Superoxide dismutase (U mg protein^{-1}). Treatment groups bearing different superscripts differ significantly ($p<0.05$). Data expressed as Mean±SE (n=10, r=3) and was estimated by One way ANOVA using Duncan's multiple range test in SPSS- 22.0

Cryopreservation can alter the spermatozoa's antioxidant components, including SOD, CAT and GPx (Lahnsteiner *et al.,* 2010; Lahnsteiner *et al.,* 2011; Osipova *et al*., 2016). According to Ighodaro and Akinloye (2018), this plays an indispensable role in the biological systems' antioxidant protective capacity against free radical attack. Ighodaro and Akinloye (2018) explained the role of these three antioxidants. They mentioned that SOD converts the superoxide radical or singlet oxygen radicals generated in tissues through metabolism to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). CAT in the peroxisomes breaks down H_2O_2 into water and molecular oxygen, consequently curtailing free radical-induced damage, whereas in mitochondria, CAT is absent. Hence, GPx reduces H_2O_2 to water and lipid peroxides to their corresponding alcohols, and therefore, the antioxidants are referred to as first-line defence antioxidants. Thus, fish fed with antioxidants had better milt quality even after cryopreservation than those not supplemented with antioxidants. Future studies integrating both antioxidant defense and oxidative damage markers will be essential to establish a comprehensive understanding of the mechanisms underlying improved cryotolerance.

Interestingly, although antioxidant enzyme activities peaked at higher vitamin E inclusion (600 mg kg^{-1}), optimal sperm performance was observed at 200 mg kg^{-1}, suggesting that maximal enzymatic activity does not necessarily correspond to improved functional outcomes. This discrepancy may reflect a threshold beyond which excessive antioxidant supplementation disrupts redox balance or exerts pro-oxidant effects, thereby emphasising the importance of optimal rather than maximal antioxidant dosing. These findings underscore the need for precise optimisation of antioxidant supplementation, as excessive inclusion may not confer additional benefits and could potentially compromise reproductive performance.

The results obtained from the current study demonstrated that dietary supplementation of antioxidant, such as vitamin C and E at appropriate concentrations significantly improves sperm quality and ovarian development in koi carp. These results suggest that, apart from supplementing antioxidants through freezing media, dietary antioxidant supplementation of broodstock represents an effective strategy for enhancing reproductive performance and improving the quality and cryosurvival of gametes.

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