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DNA damage, oxidative stress, decreased viability and motility in common carp (*Cyprinuscarpio* L.) spermatozoa induced by tryptophan, phenylalanine and cysteine amino acids during short-term storage

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Abstract: Free amino acids are found in spermatozoa and seminal plasma. Their qualitative and quantitative presence have been affected by many factors. Under in vitro storage conditions, their effects on spermatozoa in animal species and also human have been concerned over the last decades. In this study, the effects of tryptophan, phenylalanine and cysteine on DNA integrity, lipid peroxidation, viability and motility parameters in spermatozoa of the common carp (*Cyprinuscarpio*) were assessed during short-term storage. Spermatozoa were incubated at 1, 5, 25, and 50 mM of these amino acids in vitro at 4 °C in a time-dependent manner (6, 24, and 48 h). The supplementation of cysteine, phenylalanine and >5 mM of tryptophan to the incubation medium attenuated the spermatozoa motility parameters and viability. Particularly, >1 mM of cysteine completely inhibited progressive motility and decreased viability to <50%. Cysteine and phenylalanine at all concentrations significantly caused the increases in lipid peroxidation products in spermatozoa and seminal plasma, and hereby induced the percentage of DNA damage in spermatozoa. Cysteine had more detrimental effects than phenylalanine and tryptophan. Our data provide evidence that these amino acids are not beneficial for the maintaining of carp spermatozoa at 4 °C.

Keywords: DNA damage, oxidative stress, cysteine, phenylalanine, tryptophan, spermatozoa motility

1. Introduction

The availability and functionality of free amino acids (FAAs) in male reproductive system have received considerable scientific attention. Particularly, their role for spermatozoa and seminal plasma have been studied extensively in different animal species as well as in human (Chubb and Cooper, 1962; Lahnsteiner, 2009; Juyena and Stelletta, 2012; Gilany et al., 2015). They could naturally occur in spermatozoa and seminal plasma, and also their quality and quantity are species- and individual-dependent in animals including fish species. In fish, FAA are released by epithelial cells of the reproductive tracts or originated from the degradation complex protein by proteases enzymes (Setchell et al., 1967; Lahnsteiner et al., 1994; Ciereszko et al., 1998).

Because of their availability in seminal plasma of fish (Ciereszko et al.,2000), exogenous FAAs have been widely used for short- and long-term preservation (cryopreservation) of spermatozoa by adding them to extenders or cryomedia to improve spermatozoa quality (Lahnsteiner, 2009; Kledmanee et al., 2013; Kwasek et al., 2014; Li et al., 2018; Kutluyer et al., 2019; Kocabaş et al., 2019). Previous studies about the usage of exogenous FAA generally focused on spermatozoa motility, and revealed contradictory findings about the improvement of spermatozoa quality. For instance, Lahnsteiner (2009) tested the FAA in vitro at 4°C on carp and rainbow trout (*Oncorhynchus mykiss*), and reported that tryptophan treatment had no effect on motility while Kutluyer (2018) suggested that tryptophan improved motility in activation medium in rainbow trout spermatozoa. Similarly, it was found that cysteine had a negative effect on carp spermatozoa viability and motility (Lahnsteiner, 2009) while the positive effects of cysteine on the same parameters of carp spermatozoa were also reported (Kledmanee et al., 2013). These kind of inconsistent results were also observed in cryopreservation. Supplementation of cysteine to cryomedium showed no protective effect against spermatozoa motility, membrane integrity as well as reactive oxygen species in three sturgeon species (Li

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et al., 2018) while it prevented postthaw DNA damage in carp spermatozoa (Öğretmen et al., 2015). FAAs have been added ranged from 1 to 20 mM in these studies.

In this regard, particularly the effects of cysteine, phenylalanine, and tryptophan among all FAAs on spermatozoa are needed to be revealed by detailed motility and also molecular analyses. Therefore, the objective of this study was to assess the in vitro effects of different concentrations (1, 5, 25, and 50 mM) of tryptophan, phenylalanine, and cysteine amino acids on spermatozoa of carp *Cyprinuscarpio*. DNA fragmentation, motility parameters, and vitality of incubated spermatozoa at 4°C were assessed by sampling at 6, 24, and 48 hours, including lipid peroxidation levels of both spermatozoa and supernatants.

2. Materials and methods

2.1. Broodstock handling and collection of gametes

3–4-year-old common carps (n = 5) which raised at Mediterranean Fisheries Research Production and Training Institute, Antalya, Turkey for the routine production were used for the experiments. The experiments were approved by the local ethics committee (2018/E.1333483). The male fish were collected from the earthen ponds 48 h before the sampling and transported into the hatchery (22 °C) for acclimatization and fasting. Fish were injected with carp pituitary extract (Argent, USA) solved in 0.65% NaCl solution at a dose of 2 mg/kg body weight to stimulate spermiation 10 h before sampling. After being anesthetized in 0.3 mL/L of 2-phenoxyethanol, fish were removed from the water, and their genital apertures were wiped dry. Spermatozoa samples were collected by abdominal massage avoiding contamination with mucus, feces, urine or water. The samples were placed in a styrofoam box with ice and immediately transported to the laboratory for controlling motility.

2.2. Spermatozoa dilution and experimental groups

All spermatozoa samples (n = 5) exhibiting ≥90% motility were pooled and then used for the experiments. An immobilization medium composed of 75 mM NaCl, 70 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, and 20 mM Tris, pH 8.0 (Lahnsteiner et al., 1999) was used for incubation. The pooled sample was diluted with the immobilization medium containing (final concentrations) 1, 5, 25, and 50 mM of tryptophan (labeled as T1, T5, T25, and T50, respectively), phenylalanine (labeled as P1, P5, P25, and P50, respectively) and cysteine (labeled as C1, C5, C25, and C50 respectively) at 1:9 ratios. Amino acids were used as L-Tryptophan, L-Phenylalanine, and L-Cysteine (Merck, Darmstadt, Germany). Also, a control group was diluted with the immobilization medium without any amino acid. A 200 µL of the pooled sample was used for each replicate which was stored under aerobic conditions for 48 h at 4 °C. Each experimental treatment was done in 4 triplicate samples. Aliquots from the same triplicate were taken at 6, 24, and 48 h after starting storage for spermatozoa motility parameters while the other triplicates were used for assessment of DNA fragmentation, percentage of viability spermatozoa, and lipid peroxidation.

2.3. Spermatozoa quality parameters analysis

Spermatozoa quality parameters were assessed by SCA® (Sperm Class Analyzer v. 4.0.0. by Microptic S.L., Barcelona, Spain). The SCA system includes a Basler camera (Basler AG, Ahrensburg, Germany) mounted on a Nikon Eclipse 50i (Nikon Corporation, Tokyo, Japan). Three different modules of SCA were used for assessment of spermatozoa quality. The SCA Motility and Concentration module was used for determination of motility parameters and spermatozoa density. Spermatozoa samples were diluted with the immobilization medium at a final ratio of 1:4999 (v:v). For the determination of motility parameters, the spermatozoa samples were made a two-step dilution. The samples were diluted 1:99 (v:v) with the immobilization medium, and then this suspensions were diluted 1:49 (v:v) with an activating medium. The activating medium consisted of 45mM NaCl, 5mM KCl, and 30mM Tris, pH 8.0 (Perchec et al., 1995) with addition of 0.1% BSA. After intense stirring, this dilution was immediately placed into a 20 µ Leja counting chamber (Nieuw Vennep, Netherlands). Basically, the SCA settings suggested by Dziewulska et al. (2011) were applied in the current study. The minimum particle area is 1µm^2 while the maximum 30 µm^2, VAP points of 5 pixels, brightness 128, contrast 128 were set in the SCA. Spermatozoa head trajectories at 10 s postactivation were analyzed at 100× magnification. At least five recordings were recorded for each replicate. Fifty spermatozoa were analyzed in each record. Curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), linear velocity (VSL, µm/s), straightness (STR = VSL/VAP × 100), linearity (LIN = VSL/VCL × 100), and wobble (WOB = VAP/VCL) were calculated as spermatozoa motility parameters. Also, percentage of total motile spermatozoa (TM, as motile spermatozoa with VCL ≥ 20 µm/s) and progressive motile spermatozoa (PM, as motile spermatozoa with STR ≥ 80%) were calculated. The SCA Vitality module was used to reveal the percentage of alive and dead spermatozoa. For this reason, the samples were treated with a modified eosin-nigrosin stain, Bright Vit (Microptic S.L., Barcelona, Spain) which displays dead spermatozoa in red while alive spermatozoa in white. The SCA DNA Fragmentation module showed the percentage of fragmented and nonfragmented DNA of spermatozoa using by the Halosperm® kit (Halotech DNA SL, Madrid, Spain).

2.4. Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) were determined as an indicator of lipid peroxidation. TBARS contents of both spermatozoa and seminal plasma of

diluted samples were measured spectrophotometrically as basically described by Dzyuba et al. (2014). The diluted samples from all treatments were centrifuged at 5,000 × *g* at 4 °C for 10 min. The supernatants were carefully pipetted into other tubes to determine their TBARS contents. The spermatozoa pellet was diluted with the same volume of the immobilization medium (1.8 mL). These suspensions were homogenized in an ultrasonic ice bath (Bandelin electronic GmbH & Co KG, Berlin, Germany), and then centrifuged at 5,000 × *g* at 4°C for 10 min to remove debris of the cells. 250 µL of supernatants were mixed with 25 µL butylated hydroxytoluene solution (22 mg in 10 mL ethanol), 25 µL ferric chloride solution (27 mg of $FeCl_3$ - $6H_2O$ in 10 mL water), 375 µL 0.2 M glycine-hydrochloric acid buffer, pH 3.6, and 375 µL TBA reagent (0.5% TBA and 0.3% sodium dodecyl sulfate). The mixture was heated for 15 min at 100 °C in a water bath, and cooled in ice water. 0.25 mL glacial acetic acid and 0.5 mL chloroform were added to the mixture. After vigorously vortex, it was centrifuged at 1,500 × *g* for 10 min to remove the precipitate. The absorbance of the supernatant was measured at 535 nm using a 1-cm cuvette. The supernatants were diluted with the immobilization medium, when needed. The molar extinction coefficient of 1.56 × 10^5/M/cm was used to calculate the amount of malonaldehyde. All supernatants were frozen at –80 °C until used. TBARS contents of the supernatants were referred to as nmol/mL samples while TBARS contents of the spermatozoa pellet as nmoL/10^8 cells.

2.5. Statistical analysis

The results were analyzed using the statistical program SPSS 19.0 (IBM Corp., Armonk, NY, USA), and expressed as mean ± standard deviation. Percentage and metrical data were subjected to arcsin or log transformation prior to the analysis. Spermatozoa motility parameters, spermatozoa viability and lipid peroxidation levels in the treatments were analyzed by two-way ANOVA using doses of amino acids and incubation times as the main effects, and Tukey post hoc test was performed. Also, one-way ANOVA followed by Tukey post hoc test was used to clearly reveal the differences in the percentages of DNA fragmentation at the same concentration of amino acids. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Spermatozoa motility parameters

Spermatozoa density in the pooled sample was 6.2 ± 0.19 × 10^9/mL while the density in the diluted sample ranged from 1.24–1.25 × 10^8/mL. Spermatozoa velocity parameters were determined at 6, 24, and 48 h exposure of the amino acids in vitro (Table 1). The increasing concentrations of tryptophan, phenylalanine, and cysteine in time caused reduction in VCL, VAP, and VSL. However, no statistical significance was observed between the control and 1 mM of tryptophan at 6 h ($P > 0.05$). The velocity parameters measured in all treatments were significantly higher than those in 25 and 50 mM of cysteine ($P < 0.05$). Also, motility indices (STR, LIN, and WOB) and percentages (TM and PM; Table 1) in these two groups were the lowest. TM and PM values decreased dramatically in >1 mM of cysteine ($P < 0.05$), and no PM values were recorded in 5, 25 and 50 mM of cysteine at 48 h.

3.2. Spermatozoa viability, oxidative stress in spermatozoa and seminal plasma

There were significant decreases in vitality percentage with increasing tryptophan, phenylalanine, and cysteine concentration (5, 25, and 50 mM) and time duration (6, 24, and 48 h; Table 2). Spermatozoa vitality obtained from the control and 1 mM of tryptophan (97 ± 2 and 96 ± 2, respectively) were significantly higher ($P < 0.05$) than those values from 1 mM of phenylalanine and cysteine (86 ± 2 and 84 ± 3, respectively) after 6 h incubation. The lowest vitality percentages at 6, 24 and 48 h were detected in 50 mM of cysteine as 63 ± 3, 36 ± 2, and 22 ± 5 %, respectively.

Both TBARS levels in spermatozoa and seminal plasma induced by the amino acids shown particularly dose-dependent relationship regarding to which the amino acids to apply (Table 2). TBARS levels in spermatozoa from the control were measured as 0.20 ± 0.03 nmol/10^8 spermatozoa at 6 hs and 0.22 ± 0.02 nmol/10^8 spermatozoa at 24 h, showing no significant differences ($P > 0.05$). Spermatozoa TBARS levels in the control (0.33 ± 0.040 nmol/10^8 spermatozoa) were significantly increased after 48 h ($P < 0.05$). Spermatozoa TBARS levels in 1 and 5 mM of tryptophan were not different from those values measured in the control at 6, 24, and 48 h ($P < 0.05$). Also, TBARS production of spermatozoa in 1 mM of phenylalanine at 6 h (0.25 ± 0.02 nmol/10^8 spermatozoa) was not significantly increased compared to the control ($P > 0.05$). The highest TBARS levels were observed at 5≥ mM of cysteine groups at all exposure durations. Particularly, spermatozoa TBARS productions in 50 mM of cysteine at 6, 24, and 48 h were around 7, 11, and 13-fold higher than those at the same exposure durations in the control. As similar to the trend in the spermatozoa samples, TBARS levels detected in the diluted seminal plasma depended on the amino acids and their increasing doses. In the control, TBARS levels were measured as 0.05 ± 0.02, 0.07 ± 0.03, and 0.16 ± 0.03 nmol/mL samples at 6, 24, and 48 h, respectively. TBARS levels in 1 mM of tryptophan, phenylalanine, and cysteine showed no significant difference compared to the control at all exposure durations. Similarly, TBARS levels in 5 mM of tryptophan and phenylalanine were not significantly increased as compared with control and 1 mM of the amino acids after 6, 24, and 48 h; however, those levels in 5 mM of cysteine as 0.15 ± 0.03, 0.24 ± 0.04, and 0.33

Table 1. Changes in common carp (*Cyprinuscarpio* L.) spermatozoa parameters treated with tryptophan (T), phenylalanine (P), cysteine (C) at concentrations of 1, 5, 25, and 50 mM at 6, 24, and 48 h of the incubation at 4 °C.

Treatment	Time (h)	VLC	VAP	VSL	STR	LIN	WOB	TM	PM
Control	6	141 ± 4^{a}	129 ± 9^{a}	114 ± 11^{a}	89 ± 5^{a}	81 ± 7^{a}	92 ± 6^{a}	99 ± 1^{a}	95 ± 4^{a}
	24	108 ± 5^{b}	100 ± 7^{b}	79 ± 12^{b}	80 ± 9^{b}	73 ± 10^{b}	92 ± 5^{a}	85 ± 4^{b}	68 ± 4^{b}
	48	98 ± 5^{c}	90 ± 8^{c}	70 ± 10^{b}	79 ± 14^{b}	72 ± 12^{b}	92 ± 6^{a}	66 ± 5^{c}	46 ± 6^{c}
T1	6	135 ± 4^{a}	125 ± 6^{a}	106 ± 13^{a}	85 ± 11^{a}	77 ± 11^{ab}	93 ± 4^{a}	90 ± 5^{a}	87 ± 5^{a}
	24	113 ± 3^{d}	104 ± 10^{c}	81 ± 14^{bc}	78 ± 11^{ab}	72 ± 13^{abc}	93 ± 8^{a}	76 ± 3^{b}	56 ± 8^{cd}
	48	103 ± 3de	94 ± 7^{d}	58 ± 23de	61 ± 22^{b}	56 ± 20^{c}	92 ± 6^{a}	61 ± 3^{c}	49 ± 6^{cd}
P1	6	127 ± 7^{b}	117 ± 8^{ab}	86 ± 19^{bc}	73 ± 15^{ab}	68 ± 16^{abc}	92 ± 6^{a}	84 ± 2^{ab}	74 ± 4^{ab}
	24	104 ± 6de	96 ± 7^{cd}	71 ± 19^{cde}	74 ± 18^{ab}	72 ± 18^{abc}	93 ± 6^{a}	59 ± 5^{c}	56 ± 7^{cd}
	48	100 ± 3^{e}	93 ± 7^{d}	57 ± 23^{e}	61 ± 24^{b}	57 ± 23^{c}	93 ± 6^{a}	56 ± 6^{c}	48 ± 8^{cd}
C1	6	122 ± 3^{c}	115 ± 6^{b}	96 ± 13^{ab}	84 ± 9^{a}	79 ± 11^{a}	94 ± 5^{a}	84 ± 3^{ab}	75 ± 5^{ab}
	24	106 ± 4^{d}	99 ± 6^{cd}	77 ± 14^{cd}	77 ± 13^{ab}	73 ± 13^{abc}	94 ± 3^{a}	74 ± 3^{b}	65 ± 4^{bc}
	48	100 ± 4^{e}	91 ± 8^{d}	61 ± 10de	67 ± 13^{ab}	61 ± 11^{bc}	91 ± 8^{a}	63 ± 3^{c}	47 ± 5^{d}
T5	6	130 ± 6^{a}	119 ± 9^{a}	101 ± 12^{a}	85 ± 10^{a}	78 ± 10^{ab}	91 ± 6^{ab}	88 ± 2^{a}	84 ± 4^{a}
	24	110 ± 3^{b}	104 ± 5^{bc}	79 ± 12^{bc}	77 ± 13^{ab}	72 ± 12^{abcd}	94 ± 4^{ab}	67 ± 4^{bc}	53 ± 4^{b}
	48	96 ± 4^{e}	89 ± 5^{e}	55 ± 19^{def}	62 ± 20^{bc}	58 ± 19de	93 ± 4^{ab}	58 ± 4^{cd}	47 ± 3^{bc}
P5	6	113 ± 5^{b}	108 ± 8^{b}	92 ± 12^{ab}	86 ± 10^{a}	82 ± 10^{a}	95 ± 5^{a}	78 ± 4^{ab}	73 ± 6^{a}
	24	100 ± 4^{d}	94 ± 7de	71 ± 15^{cd}	76 ± 16^{ab}	71 ± 15^{abcd}	93 ± 5^{ab}	60 ± 4^{c}	55 ± 6^{b}
	48	89 ± 3^{f}	79 ± 7^{f}	54 ± 17^{ef}	68 ± 19^{abc}	60 ± 18^{cde}	88 ± 7^{b}	57 ± 4^{cd}	51 ± 5^{b}
C5	6	105 ± 3^{c}	100 ± 5^{cd}	80 ± 11^{bc}	81 ± 12^{a}	77 ± 10^{abc}	95 ± 3^{a}	68 ± 4^{bc}	58 ± 4^{b}
	24	89 ± 3^{f}	80 ± 8^{f}	56 ± 18de	70 ± 21^{abc}	63 ± 20^{bcde}	89 ± 8^{ab}	48 ± 4^{d}	35 ± 6^{c}
	48	80 ± 2^{g}	72 ± 6^{f}	39 ± 14^{f}	54 ± 19^{c}	49 ± 18^{e}	90 ± 7^{ab}	22 ± 4^{e}	0 ± 0
T25	6	113 ± 5^{a}	108 ± 5^{a}	81 ± 15^{a}	75 ± 13^{a}	72 ± 13^{a}	96 ± 3^{a}	78 ± 3^{a}	69 ± 4^{a}
	24	99 ± 4^{c}	91 ± 7^{b}	65 ± 14^{ab}	71 ± 16^{ab}	66 ± 15^{a}	92 ± 4^{a}	56 ± 8^{b}	47 ± 6^{c}
	48	85 ± 3^{e}	77 ± 6^{cd}	56 ± 16^{bc}	72 ± 19^{ab}	65 ± 19^{a}	90 ± 7^{a}	53 ± 4^{b}	42 ± 5^{cd}
P25	6	104 ± 3^{b}	97 ± 8^{b}	80 ± 17^{a}	82 ± 16^{a}	77 ± 16^{a}	93 ± 5^{a}	70 ± 3^{a}	62 ± 6^{ab}
	24	91 ± 2^{d}	81 ± 10^{c}	57 ± 21^{bc}	71 ± 25^{ab}	63 ± 24^{a}	89 ± 11^{a}	52 ± 3^{bc}	48 ± 5^{bc}
	48	80 ± 3^{f}	70 ± 6de	46 ± 17^{c}	66 ± 24^{ab}	58 ± 21^{b}	88 ± 5^{a}	41 ± 3^{cd}	39 ± 3
C25	6	89 ± 3de	81 ± 7^{c}	66 ± 12^{ab}	82 ± 15^{a}	74 ± 14^{a}	91 ± 7^{a}	55 ± 5^{b}	48 ± 7^{bc}
	24	71 ± 3^{g}	62 ± 9^{e}	43 ± 17^{c}	69 ± 24^{ab}	61 ± 24^{a}	88 ± 10^{a}	31 ± 5^{d}	29 ± 5^{d}
	48	40 ± 4^{h}	30 ± 7^{f}	16 ± 8^{d}	51 ± 15^{b}	39 ± 17^{b}	74 ± 14^{b}	16 ± 4^{e}	0 ± 0
T50	6	103 ± 3^{a}	94 ± 7^{a}	63 ± 16^{a}	67 ± 15^{ab}	61 ± 15^{ab}	91 ± 7^{a}	66 ± 6^{a}	57 ± 5^{a}
	24	90 ± 2^{b}	82 ± 5^{b}	64 ± 18^{a}	78 ± 22^{ab}	72 ± 20^{b}	92 ± 6^{a}	55 ± 3^{ab}	45 ± 8^{ab}
	48	70 ± 2^{d}	58 ± 9^{d}	39 ± 15^{c}	68 ± 23^{ab}	56 ± 22^{ab}	82 ± 12^{a}	42 ± 6^{b}	35 ± 8^{b}
P50	6	90 ± 3^{b}	82 ±5^{b}	58 ±20^{ab}	71 ± 24^{ab}	65 ± 23^{b}	92 ± 6^{a}	54 ± 5^{ab}	45 ± 5^{ab}
	24	80 ± 4^{c}	69 ± 9^{c}	54 ± 16^{abc}	79 ± 22^{ab}	68 ± 20^{b}	86 ± 9^{a}	44 ± 5^{b}	39 ± 3^{b}
	48	64 ±4^{e}	56 ± 8^{d}	43 ± 13^{bc}	76 ± 17^{ab}	67 ± 20^{b}	87 ± 12^{a}	42 ± 3^{b}	34 ± 4^{b}
C50	6	79 ± 3^{c}	72 ± 6^{c}	62 ± 10^{a}	86 ± 14^{a}	78 ± 15^{a}	90 ± 8^{a}	50 ± 5^{b}	42 ± 6^{ab}
	24	60 ± 3^{f}	49 ± 9^{d}	40 ± 15^{c}	78 ± 20^{ab}	66 ± 25^{b}	82 ± 15^{a}	19 ± 5^{c}	14 ± 4^{c}
	48	28 ± 4^{g}	19 ± 4^{e}	11 ± 4^{d}	60 ± 13^{b}	42 ± 15^{b}	69 ± 17^{b}	15 ± 4^{c}	0 ± 0
Two-way ANOVA P-value	Dose	0.000	0.000	0.000	0.008	0.001	0.000	0.000	0.000
	Time	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Dose × time	0.000	0.000	0.030	0.054	0.212	0.000	0.941	0.740

Data within the same concentration are superscripted by the different lower case letter are significantly different ($P < 0.05$).

Table 2. Effect of tryptophan (T), phenylalanine (P), cysteine (C) at concentrations of 1, 5, 25, and 50 mM on common carp spermatozoa viability and TBARS content at 6, 24, and 48 h of the incubation at 4 °C.

Treatment	Time (h)	Viability (%)	TBARS in spermatozoa (nmoL/10^8 cells)	TBARS in seminal plasma (nmol/mL samples)
Control	6	97 ± 2^{a}	0.20 ± 0.03^{b}	0.05 ± 0.02^{b}
	24	92 ± 3^{a}	0.22 ± 0.02^{b}	0.07 ± 0.03^{b}
	48	85 ± 3^{b}	0.33 ± 0.04^{a}	0.16 ± 0.03^{a}
T1	6	96 ± 2^{a}	0.19 ± 0.02^{d}	0.06 ± 0.01^{cd}
	24	90 ± 3^{ab}	0.24 ± 0.02^{cd}	0.10 ± 0.01^{bc}
	48	81 ± 3^{cd}	0.32 ± 0.04^{ab}	0.18 ± 0.02^{a}
P1	6	86 ± 2^{bc}	0.25 ± 0.02^{cd}	0.06 ± 0.02^{d}
	24	82 ± 1^{cd}	0.29 ± 0.02^{bc}	0.10 ± 0.01^{bc}
	48	74 ± 2^{e}	0.35 ± 0.02^{ab}	0.16 ± 0.02^{a}
C1	6	84 ± 3^{bc}	0.29 ± 0.02^{bc}	0.08 ± 0.01^{bcd}
	24	81 ± 3^{cd}	0.31 ± 0.03^{ab}	0.11 ± 0.01^{b}
	48	75 ± 2^{de}	0.36 ± 0.02^{a}	0.19 ± 0.01^{a}
T5	6	95 ± 1^{a}	0.24 ± 0.02^{f}	0.07 ± 0.01^{e}
	24	86 ± 4^{b}	0.28 ± 0.02^{ef}	0.12 ± 0.02^{de}
	48	78 ± 3^{c}	0.35 ± 0.03^{cde}	0.20 ± 0.02^{bc}
P5	6	78 ± 2^{c}	0.31 ± 0.02^{def}	0.08 ± 0.01^{e}
	24	72 ± 2^{cd}	0.35 ± 0.02^{cde}	0.15 ± 0.01^{cd}
	48	67 ± 1^{d}	0.39 ± 0.02^{bcd}	0.20 ± 0.01^{bc}
C5	6	79 ± 3^{c}	0.41 ± 0.03^{bc}	0.15 ± 0.03^{cd}
	24	66 ± 3^{d}	0.47 ± 0.05^{b}	0.24 ± 0.04^{b}
	48	54 ± 4^{e}	0.67 ± 0.06^{a}	0.33 ± 0.02^{a}
T25	6	82 ± 3^{a}	0.31 ± 0.02^{f}	0.11 ± 0.02^{f}
	24	74 ± 3^{b}	0.38 ± 0.03^{f}	0.15 ± 0.02^{ef}
	48	68 ± 1^{bc}	0.54 ± 0.03^{de}	0.26 ± 0.02^{cd}
P25	6	70 ± 2^{b}	0.34 ± 0.01^{f}	0.14 ± 0.02^{ef}
	24	62 ± 2^{cd}	0.41 ± 0.02^{ef}	0.21 ± 0.03^{de}
	48	56 ± 2^{d}	0.62 ± 0.03^{d}	0.31 ± 0.03^{c}
C25	6	69 ± 2^{bc}	0.92 ± 0.06^{c}	0.39 ± 0.03^{b}
	24	45 ± 4^{e}	1.20 ± 0.08^{b}	0.58 ± 0.03^{a}
	48	32 ± 4^{f}	1.67 ± 0.10^{a}	0.65 ± 0.05^{a}
T50	6	80 ± 2^{a}	0.31 ± 0.02^{e}	0.16 ± 0.02^{e}
	24	67 ± 3^{bc}	0.40 ± 0.02^{e}	0.21 ± 0.02^{de}
	48	57 ± 4^{d}	0.63 ± 0.06^{de}	0.36 ± 0.02^{cd}
P50	6	71 ± 3^{b}	0.36 ± 0.04^{e}	0.22 ± 0.03^{de}
	24	61 ± 3^{cd}	0.48 ± 0.04^{e}	0.32 ± 0.03^{cde}
	48	44 ± 4^{e}	0.87 ± 0.09^{d}	0.42 ± 0.04^{c}
C50	6	63 ± 3^{bcd}	1.39 ± 0.14^{c}	0.42 ± 0.03^{c}
	24	36 ± 2^{e}	2.53 ± 0.25^{b}	0.71 ± 0.07^{b}
	48	22 ± 5^{f}	4.25 ± 0.28^{a}	1.22 ± 0.13^{a}
Two-way ANOVA P-value	Dose	0.000	0.000	0.000
	Time	0.000	0.036	0.000
	Dose × time	0.342	0.287	0.327

Data within the same concentration are superscripted by the different lower case letter are significantly different ($P < 0.05$).

± 0.02 nmol/mL samples at 6, 24, and 48 h, respectively, were higher than those in the control at the same exposure durations.

3.3. DNA fragmentation

Similar to oxidative stress, percentages of DNA fragmentation were increased in amino acid, dose and time-dependent manner. The percentages of DNA fragmentation in 1 and 5 mM of tryptophan at 6, 24, and 48 h, in 1 mM of phenylalanine and cysteine were not different from those percentages measured in the control ($P < 0.05$). After 48 hours, DNA fragmentation was 3 ± 1% in the control whereas 34 ± 3% was detected in 50 mM of cysteine, which was the highest value measured in all treatments (Figure 1).

4. Discussion

Even though their roles and functions remain unclear, FAAs in both spermatozoa and seminal plasma of animal species including fish and human have been detected in many studies. In fish, it is reported that the reproductive system ducts secrete these amino acids (Lahnsteiner et al., 1994). They might support the regulation of seminal plasma osmolality and spermatozoa vitality, and also could be an alternative energy source for spermatozoa during motility (Billard and Menezo, 1994; He and Woods, 2003; Lahnsteiner, 2010). The quality and quantity of them show individual-dependent variations. Also, they are affected by proteolysis capacity of proteins in seminal plasma, age and diet of fish, and environmental conditions as well (Lahnsteiner et al., 1994; Tantikitti and March, 1995; Ciereszko et al., 1998). An increased in only one amino acid ratio in the diet (such as lysine or arginine) could change the FAA composition of semen in fish (Kwasek et al., 2014; Pourkhazaei et al, 2017). FAAs from damaged spermatozoa are released to seminal plasma, and could change their composition in it (Schmehl et al., 1987). Moreover, this composition is under the influence of seasonality (Shaliutina-Kolešová et al., 2016).

FAAs have been tested used for spermatozoa studies in different mammalian species, such as ram, goat, buffalo bull and stallion (Sanchez-Partida et al., 1992; Trimeche et al., 1999; Kundu and Das, 2001; Bucak et al., 2009; Tuncer et al., 2010; Topraggaleh et al., 2014), and also in poultry such as chicken (Santiago-Moreno et al., 2019). Although some contradictory results for spermatozoa of fish species, the positive and negative effects of FAAs at defined concentrations were reported more clearly for spermatozoa of mammalian species than of fish. This could be due to metabolic and functional differences of FAA between mammalian and fish semen.

The FAA compositions of semen in carp, rainbow trout, gilthead sea bream *Sparusaurata*, and perch *Percafluviatilis* have been determined. It was shown that the qualitative composition of them could be similar up to only 52% (Billard and Menezo, 1984; Lahnsteiner, 2009; 2010). The detection of FAA has shown differences among species, and even could be varied from spermatozoa to seminal plasma of a same species. For istance, cysteine and phenylalanine were detected in seminal plasma of perch whereas they were not found in spermatozoa of the same species. Additionally, tryptophan was found in spermatozoa and seminal plasma of perch and carp but not in those of gilthead sea bream (Billard and Menezo, 1984; Lahnsteiner, 2010). Stejskal et al. (2008) reported the content of cysteine in semen of Siberian sturgeon *Acipenserbaerii*, sterlet *A. ruthenus*, pikeperch *Sander lucioperca* and perch. The cysteine concentrations in semen of pikeperch and perch were 5–6 fold more than those in the Acipenser species. They reported the cysteine concentration in perch semen as 2.31 ± 0.35 mM (n = 2) while Lahnsteiner (2010) found it as 0.57 ± 0.81 mM (n = 6). Moreover, the cysteine concentration in seminal plasma of carp was marginally detected as 5.77 ± 2.62 µM (n = 7) (Lahnsteiner, 2010). Cysteine just like other FAA in semen exhibits a great variation depending on species. Additionally, the high standard deviation values

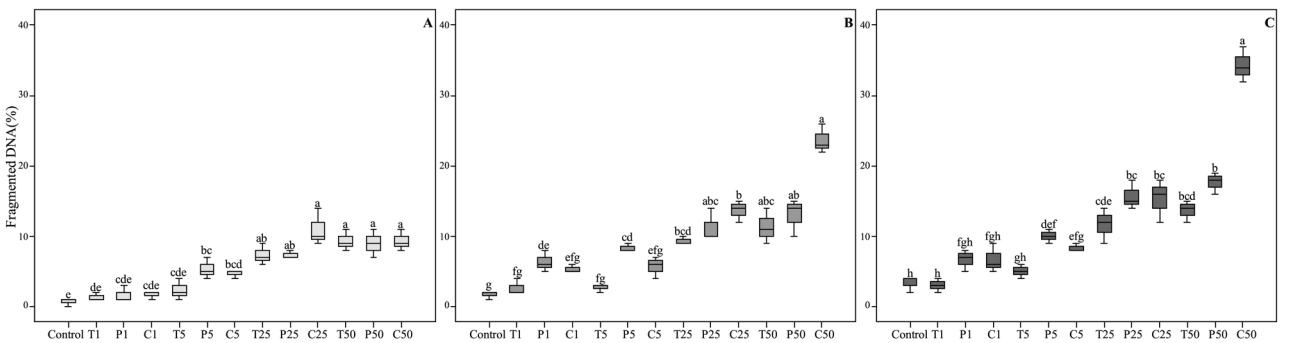

Figure 1. Effect of tryptophan (T), phenylalanine (P), cysteine (C) at concentrations of 1, 5, 25, and 50 mM on DNA fragmentation of common carp (*Cyprinuscarpio* L.) spermatozoa at 6 (a), 24 (b), and 48 (c) h.

of these data also imply a great variability, which could arise due to small sample size or be actually an individual-dependent manner. Therefore, after the addition of FAAs to a medium for spermatozoa processing techniques, final concentrations of them in this medium could remain unclear. This might be main reason for the inconsistent results among fish studies.

In the current study, the supplementation of cysteine to the medium showed the negative effects on carp spermatozoa motility parameters, viability, and increased lipid peroxidation and DNA damage. These negative effects raised with the increasing cysteine concentration. Similarly, Li et al. (2018) reported that cysteine concentration up to ~10 mM has no effect on spermatozoa quality parameters, but >10 mM of cysteine caused the lower postthaw spermatozoa motility, membrane integrity, and fertilization rate for spermatozoa of three sturgeon species. On the contrary, the increasing cysteine concentration (up to 20 mM) was correlated with the higher motility and fertilization rate, the lower DNA damage in cryopreserved carp spermatozoa (Öğretmen et al., 2015). Kledmanee et al. (2013) reported that 1 mM of cysteine improved percentage and duration of carp spermatozoa motility at 4°C, and also 2 mM of cysteine caused the higher spermatozoa viability after 48 h incubation at 4 °C compared to the control. In consistent with these results, Lahnsteiner (2009) assessed the effects of 1.3, 2.5, and 5 mM of cysteine on carp spermatozoa, and suggested that cysteine reduced spermatozoa progressive motility and viability, particularly this reduction was significant after 24 h incubation. This result is in agreement with the results of the current study. Moreover, our results revealed that cysteine caused not just reduction in spermatozoa moility and viability but also increase lipid peroxidation and DNA fragmentation. The inconsistent results among these studies could be caused by physiological and methodological reasons. Kledmanee et al. (2013) sampled 0.5–1 kg of carp while around 3 kg carp breeders were used in the current study. Also, Öğretmen et al. (2015) treated spermatozoa samples with cryomedium containing cysteine in a short time (for only 5 min at 4°C, and then for 10 min at a height of 3 cm above liquid nitrogen surface) when tested cysteine for cryopreservation of carp spermatozoa.

Cysteine is one of the most reactive amino acid among them, and also has a sulfhydryl group at its side chain which possesses a strong tendency to chemical bonding. The uptake cysteine by cells is increased by extracellular and intracellular availability of it, and the various pathways in cell membrane participate in its transportation (Yin et al., 2016). Therefore, the addition of exogenous cysteine to medium could occupy spermatozoa membrane excessively, and increase metabolic activity. This could be the main reason that the high lipid peroxidation was detected in carp spermatozoa treated with cysteine in the current study. The increase in lipid peroxidation also indicates the production of reactive oxygen species which could induce DNA fragmentation (Gazo et al., 2015). Moreover, the cytotoxicity of cysteine is very well explained in rat and rabbit when its concentration is above the threshold (Lehmann, 1987; Garcia and Stipanuk, 1992). Indeed, chronically high levels of cysteine in blood plasma have been related to common diseases in human (Heafield et al., 1990; Bradley et al., 1994; Özkan et al., 2002).

In the current study, it was observed that phenylalanine, similarly cysteine, had the negative effects on all assessed parameters in carp spermatozoa. However, cysteine caused more lipid peroxidation products, especially in spermatozoa, and DNA fragmentation than phenylalanine caused. Lahnsteiner (2009) reported that 5 mM of phenylalanine has no effects within 1 h of incubation at 4 °C, but decreased progressive motility after 1 h. In the current study, the negative effects of cysteine and phenylalanine at all concentrations on carp spermatozoa motility were observed while 1 and 5 mM of tryptophan showed no significant differences with the control group. However, the higher tryptophan concentrations affected negatively spermatozoa parameters, and caused the higher lipid peroxidation products compared to the control group. Similarly, Lahnsteiner (2009) also reported that tryptophan up to 5 mM had no effects on spermatozoa motility. The negative effects of aromatic amino acids, such as tryptophan and phenylalanine on viability bull spermatozoa have been reported (Macmillan et al., 1972). Lapointe and Sirard (1998) represented that cysteine (~2 mM) and phenylalanine (~1.5 mM) inhibited spermatozoa viability after incubation for 6 h at 37 °C. They also reported that tryptophan (~1.2 mM) showed no difference compared to the control. The similar tendencies of these amino acids have been also found in carp spermatozoa in the current study.

Due to its structure, spermatozoa membrane is highly sensitive to production of hydrogen peroxide and ammonia. When they are excessive, they could damage the integrity of membrane which is a limiting factor for spermatozoa motility (Klebanoff and Smith, 1970). Regarding to these oxidative stress agents, the detrimental effects of FAA on spermatozoa have been established well for mammalian spermatozoa. The amino acid oxidase is released by dead (or being dead during incubation) spermatozoa to medium. This enzyme dehydrogenates and deaminates FAA, and thus hydrogen peroxide and ammonia are formed excessively in medium (Griveau et al., 1995; LapointeandSirard, 1998). According to the results of the current study, a similar mechanism has been proposed for carp spermatozoa in terms of cysteine, phenylalanine and partially tryptophan.

In conclusion, the supplementations of cysteine, phenylalanine and >5 mM of tryptophan to the in vitro incubation medium at 4 °C attenuated the spermatozoa velocity parameters and viability. Moreover, particularly cysteine and phenylalanine caused the increases in lipid peroxidation products in both spermatozoa and seminal plasma, and hereby induced damage of spermatozoa DNA. These negative effects have shown concentration- and time-dependent manners. Our data provided the evidence that these amino acids are not useful for the maintaining of carp spermatozoa at 4 °C. Additionally, cysteine seemed to have more detrimental effects than phenylalanine and tryptophan.

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Conflict of interest
Authors declare no conflict of interest.

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