# Ameliorative role of *trans*-ferulic acid on induced oxidative toxicity of rooster semen by $\beta$-cyfluthrin during low temperature liquid storage

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**ABSTRACT** Current study was designed to evaluate the effects of $\beta$-cyfluthrin, as a toxicant substance, and *trans*-ferulic acid (*trans*-**FA**), as a protective agent, on different parameters of rooster semen upon liquid storage. For this purpose, semen samples of roosters (Ross 308, n = 10, 32-wk-old) were collected twice a week. Good quality samples (≥70% progressive motion) were diluted, pooled and then divided for the purposes of the study. In the first experiment, motility of spermatozoa was evaluated following exposure to different concentrations of $\beta$-cyfluthrin (1, 2.5, 5, 10, 20, 40, and 80 $\mu$M) at 0, 24, and 48 h of storage. In the second experiment, constant doses of $\beta$-cyfluthrin (10 $\mu$M) alone or in combination with *trans*-FA (10, 25 mM) were assessed on motility and viability of spermatozoa at 0, 24, and 48 h time points. Moreover, amounts of malondialdehyde (**MDA**), total antioxidant capacity (**TAC**), total nitrate-nitrite, total hydroperoxide (**HPO**), and activity of superoxide dismutase (**SOD**) were evaluated in the homogenate of spermatozoa-diluent at studied time points. Results of the first experiment showed that amounts of $\beta$-cyfluthrin greater than 5 $\mu$M, significantly reduced the motility of spermatozoa at 24 and 48 h of storage ($P < 0.05$). The second experiment demonstrated that, *trans*-FA especially at 10, 25 mM doses restored the motility and viability of spermatozoa compared to $\beta$-cyfluthrin treated group ($P < 0.05$). Amounts of MDA (10, 25 mM), hydroperoxide (10, 25, and 50 mM), and nitrate-nitrite (10, 25, and 50 mM) were lower and TAC (10 and 25 mM) were greater in *trans*-FA + $\beta$-cyfluthrin treated groups compared to $\beta$-cyfluthrin alone treated samples ($P < 0.05$). However, activity of SOD did not show significant changes by the treatment ($P > 0.05$). It seems that *trans*-FA could ameliorate toxic effect of $\beta$-cyfluthrin via reduction of peroxidative (as evident by measurement of MDA) and nitrosative (as evident by measurement of nitrate-nitrite) reactions over cold preservation of rooster semen.

**Key words:** cyfluthrin, *trans*-ferulic acid, rooster, semen

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## INTRODUCTION

Global poultry production has grown dramatically in recent decades. Developments in nutrition, breeding, and genetic selection of new breeds have played an important role in these achievements. As a result, selection for rapid growth in broiler breeders leads to lower fertility (Reddy, 1995). Therefore, it is imperative to maximize reproductive potential of broiler breeders, especially roosters. In this regard, poultry breeders use various methods of the dilution and storage of rooster semen to increase reproductive performance of birds. Particularly, liquid storage of semen is used to decrease spermatozoa metabolism and increase its viability over period of storage (Fattah et al., 2017). Ferulic acid (**FA**), a natural phenolic component, presents in free and conjugated forms in plants. Two amino acids, phenyl alanine and tyrosine, are involved in the formation of FA in plants through shikimate pathway. It is known to possess broad physiological activities such as antioxidative (direct or indirect), antiallergic, anticarcinogenic, hepatoprotective, antimicrobial, metal chelative, anti-inflammatory as well as modulation roles in cell signaling and gene expression (Kumar and Pruthi, 2014). Indirect antioxidant characteristic of FA may be through activation of nuclear factor erythroid 2-like2 pathway (Li et al., 2012). Previous study revealed that supplementation of pig feed with FA improved meat quality and antioxidant activity (Li et al., 2015). It was shown that 2 isomers of FA, trans-FA and cis-FA, scavenge free radicals and inhibit peroxidation of lipids



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(Srinivasan et al., 2007). The trans-FA is more abundant in plant cells and its separation is easier than the other form (Hartley and Jones, 1977).

Nowadays, pyrethroids are the most widely used class of pesticides, applied in agriculture and livestock production (Corcellas et al., 2015). It was documented that 25% of human habitations were polluted with cyfluthrin (Deziel et al., 2015). In poultry farms, pyrethroids are widely used to control the vectors and prevention and treatment of ectoparasitic (Chernaki-Leffer et al., 2013; Rezende et al., 2013). Following usage of pyrethroids in poultry farms, they can be absorbed and mobilized to different tissues and eggs (Goetting et al., 2011). $\beta$-cyfluthrin, a neurotoxin, is classified as a type II pyrethroids. This class of insecticide is used extensively in poultry farms due to its relative safety for target animals (Addy-Orduna et al., 2011). Axon sodium channels of insects are 100 times more sensitive to toxic effects of pyrethroids than those of mammals (Ecobichon, 2001). Also, pyrethroids act on some calcium and chloride channels which may be linked to their toxicity (Soderlund et al., 2002). Toxic effects of cyfluthrin have been previously documented in human and animals' reproduction. A research conducted in Brazil revealed that pyrethroids residues were found in 79% of the chicken eggs sampled from commercial farms and home production (Parente et al., 2017). The mentioned study also indicated that, 33% of examined samples had pyrethroids residues greater than the maximum residue limits. Reports showed that occupational exposure to pyrethroid may cause oxidative stress, immunological disturbances, and finally gene mutations (Okda et al., 2017). Moreover, other reports revealed that nonoccupational exposure to pyrethroid were associated with damages to DNA of spermatozoa and poor quality of semen (Ji et al., 2011; Han et al., 2017). Pregnancy loss and disorders of male sex hormones following cyfluthrin toxicity have also been reported (Cox, 1994). Moreover, some differences were found in the toxicity of $\beta$-cyfluthrin among bird species. In this regard, canaries were more sensitive to the toxic effects of $\beta$-cyfluthrin than the other birds, while shiny cowbirds and eared doves were practically resistant (Addy-Orduna et al., 2011). Limited knowledge is available about the effects of cyfluthrin and FA on rooster semen quality following in vivo and in vitro studies. The present in vitro study was designed to investigate the varying doses of $\beta$-cyfluthrin (as one of the wieldy-used insecticide in poultry industries) on motility of rooster spermatozoa during storage at 4°C (first object). After choosing the mild toxic dose of $\beta$-cyfluthrin, we tried to find out if concurrent supplementation with trans-FA could protect quality of rooster semen against $\beta$-cyfluthrin-induced toxicity over child storage.

## MATERIALS AND METHODS

### Husbandry and Semen Collection

A total of 10 boiler breeder males (Ross 308) of 32 wks old were included in the present study. Birds were kept in controlled environmental houses (13L: 11D, light density: 30−60 lux, 22°C) and maintained under guidelines of the Animal care of the Urmia University (IR-UU-AEC-3/PD/343). Roosters were supplied with a standard diet (2,700 Kcal metabolizable energy, 11.5% crude protein). Adaptation period lasted for three weeks. In the period of adaptation, roosters were trained with dorso-abdominal massage for semen collection process (Lake, 1957). Semen sample was collected twice a week into a graduated collection tube. Collected semen was inspected to eliminate samples with debris, urine, or excessive particulate matter. Volume of each sample was measured and recorded.

### Semen Evaluation and Allocation of Experimental Groups

Samples were transferred to the laboratory within 10 min after collection and to avoid thermal shock, semen samples were kept in a jacket after collection and during transportation to the laboratory. Motility of samples was evaluated under phase contrast microscope (Olympus, BX 41, Japan). Semen samples with greater than 70% forward progressive motility was diluted with a commercial poultry semen preservation medium (Ref. No. 020070, IMV Technologies, France) at 1:1 (v/v) ratio. Diluted samples from different roosters were pooled (equal volume of each sample) and after counting of spermatozoa, the pooled sample was diluted with the mentioned preservation medium at a final concentration of $1 \times 10^9$ spermatozoa per mL and used for the purposes of the present study.

In the first experiment, semen was exposed to 0, 1, 2.5, 5, 10, 20, 40, and 80 $\mu$M concentration of $\beta$-cyfluthrin (PESTANAL, 46003, Sigma-Aldrich, St Louis, MO) dissolved in dimethyl sulfoxide (**DMSO**; CARLO ERBA, France). The stock solution of $\beta$-cyfluthrin (20, 10, 5, 2.5, 1.25, 0.625, and 0.25 mM) dissolved in DMSO was prepared and treated samples received equal volume of DMSO dissolved $\beta$-cyfluthrin (4 $\mu$L in 1000 $\mu$L sample) with different concentration of $\beta$-cyfluthrin. A diluted sample was set as negative control group to compare the results with solvent (DMSO) and $\beta$-cyfluthrin treated groups at each run. After exposure, motility of spermatozoa was evaluated (Ommati et al., 2013) under phase contrast microscope (Olympus, BX 41) equipped with a warm stage at 0, 24, and 48 h of storage at 4°C. The total number of 54 samples in 6 sampling days (8, 9, 9, 9, 10, 9, samples in each sampling days) were used in the first experiment.

In the second experiment, diluted samples were exposed to $\beta$-cyfluthrin (10 $\mu$M) alone or supplemented concurrently with 10, 25, 50 mM of *trans*-FA. The stock solution of 10, 5, and 2 mM of *trans*-FA was prepared by dissolving appropriate amounts of *trans*-FA in DMSO. By adding 5 $\mu$L of 10, 5, and 2 mM *trans*-FA in 1,000 $\mu$L sample, the subjected amounts of *trans*-FA were 50, 25, and 10 mM, respectively. Negative control sample did not receive any treatment. Moreover, the

control sample received DMSO as solvent of *trans*-FA and $\beta$-cyfluthrin. The amounts of DMSO in treated samples were less than 1% of final volume. All experimental samples were kept at 4°C. Motility (forward progressive motility) and viability of semen samples were determined at 0, 24 and 48 h poststorage. Furthermore, amounts of total antioxidant capacity (**TAC**), malondialdehyde (**MDA**), total nitrate-nitrite, total hydroperoxide (**HPO**), and activity of superoxide dismutase (**SOD**) were measured in spermatozoa-diluent homogenate at 0, 24, and 48 h storage time points.

To evaluate the spermatozoa progressive motility, sample was diluted with extender (Ref. No. 020070, IMV Technologies) at a rate of $25 \times 10^6$ spematozoa per mL. Then, 10 $\mu$L of sample placed between a prewarmed slide and a coverslip and examined subjectively under phase contrast microscope (Olympus, BX 41). The average of 15 to 20 microscopic field was assessed to determine motility characteristics. The motion was evaluated based on a previously published article (Ommati et al., 2013). The total number of 84 samples in 9 sampling days (9, 10, 9, 10, 9, 9, 10, 9, 9 samples in each sampling days) were used in the second experiment.

Semen samples were stained with eosin-nigrosine solution to evaluate their viability (Bakst and Cecil, 1997). For this purpose, 200 cells per slide were examined using a light microscope (1,000 ×, Zeiss, Germany). Live and dead spermatozoa were stained colorless and pink, respectively.

### Measurement of Oxidative and Nitrosative Stress Indices and Antioxidant Levels in the Spermatozoa-Diluent Homogenate of Samples

After evaluation of motility and viability, the remainder of sample (spermatozoa plus diluent) for each treated group (at each time point) was homogenized using an electronic homogenizer (T10 Basic, IKA, Werke GmbH & Co. KG, Staufen, Germany) at 5,000 to 6,000 *g* for 3 min at the laboratory temperature. The obtained homogenate of spermatozoa-diluent was kept in the freeze temperature (−20°C) until biochemical assessment.

Amounts of MDA were assessed using the method described by Stern et al., (2010). In brief, the reagent was mixed with sample in a glass tube and incubated for 15 min at boiling water. After centrifugation (2,500 g, for 15 min), the absorbance of upper layer was read at 535 nm using a spectrophotometer (NOVASPEC II, Pharmacia LKB Biochrom Ltd, Cambridge, UK). Results were shown as $\mu$mol/g protein. The data were normalized according to the amounts of protein.

Values of TAC in samples were measured by the method described previously (Koracevic et al., 2001) with some modification. Briefly, sample (10 $\mu$L) was mixed with 490 $\mu$L phosphate buffer saline, 500 $\mu$L sodium benzoate, 1,000 $\mu$L acetic acid and 200 $\mu$L complex of Fe-EDTA and hydrogen peroxide, respectively. Then, tubes were incubated in a water bath (at 37°C) for 60 min. Then, thiobarbituric solution was added to the tubes and incubated at 100°C for 10 min. Finally, the optical density was recorded by a spectrophotometer (Pharmacia, Pharmacia LKB, NOVASPEC II) at the wave length of 532 nm. Amounts of TAC were expressed as mmol/g protein.

The nitrate-nitrite values in the semen were measured based on the Griess reaction (Green et al., 1982). After addition of prepared Griess reagent with the sample in 96-well plates, the plates were incubated in a dark place at laboratory temperature for 15 min. After completion of reaction, the absorbance of the solution was read by ELISA reader (DANA, Iran) at 540 nm (reference wavelength at 630 nm). Different concentrations of sodium nitrate were used to prepare the standard plot. After normalization of total nitrate-nitrite amounts by measurement of protein, the results reported as $\mu$mol/g protein in the samples.

The SOD activity of the samples was measured according to the pyrogallol oxidation method, which is reported by Marklund and Marklund (1974). Briefly, the absorbance following enzymatic reaction was recorded at two time points (1.5 and 3.5 min) after pyrogallol addition using a spectrophotometer (Pharmacia, Pharmacia LKB, NOVASPEC II). The data was normalized according to the amounts of protein in the sample, and finally reported as unit/mg protein.

Total HPO was quantified according to a previously published protocol (Nouroz Zadeh et al., 1995). The reagent was mixed with the sample in a glass tube and incubated for 30 min at laboratory temperature in dark condition. Following reaction, the absorbance of the generated solution was read and recorded at 560 nm wave length using a spectrophotometer (Pharmacia, Pharmacia LKB, NOVASPEC II). Different concentrations of hydrogen peroxide were used to prepare the standard plot. The amounts of HPO were reported as $\mu$mol/g protein in the samples.

Amount of total protein was determined by the Bradford method (Bradford, 1976). In brief, Bradford reagent and stock solution of bovine serum albumin were prepared accordingly. Subsequently, samples were mixed with the Bradford reagent and the absorbance of the generated solution was read at 595 nm wave length after 15 min incubation in laboratory temperature. Amounts of total protein were calculated using a standard curve developed for this purpose.

### Statistical Analysis

Values of spermatozoa motility and viability and the biochemical assays such as amounts of MDA, TAC, HPO, SOD, and nitrate-nitrite were exhibited as the mean ± SEM. Percentage data were subjected to arcsine transformation. One-way ANOVA coupled with Tukey's post hoc test was used to compare means of the variables recorded among groups in each time point.

**Table 1.** Progressive motility of rooster spermatozoa (%) following exposure to different concentrations of β-cyfluthrin and stored for various time points at 4°C.

| Treatment groups | Time of storage (h) | | |
|---|---|---|---|
| | 0 | 24 | 48 |
| Negative control | 66.72 ± 3.21 | 42.06 ± 2.79 | 20.11 ± 2.74 |
| Control | 68.13 ± 2.91 | 41.70 ± 2.15 | 21.10 ± 3.03 |
| β-cyf 1 μM | 68.50 ± 3.14 | 42.22 ± 2.51 | 22.19 ± 2.68 |
| β-cyf 2.5 μM | 67.11 ± 3.79 | 38.39 ± 3.03 | 17.21 ± 3.19 |
| β-cyf 5 μM | 65.19 ± 3.17 | 37.27 ± 1.96 | 15.11 ± 2.80* |
| β-cyf 10 μM | 65.41 ± 2.80 | 30.85 ± 2.19 * | 10.47 ± 1.30* |
| β-cyf 20 μM | 48.33 ± 3.58* | 17.37 ± 2.76* | 1.59 ± 1.02* |
| β-cyf 40 μM | 26.86 ± 2.11* | 0 * | 0 * |
| β-cyf 80 μM | 5.12 ± 1.36* | 0 * | 0 * |

*Indicate significant difference ($P < 0.05$) with control and negative control groups.

Repeated Measure of ANOVA was used to analyze changes of variables of each group over time points. Statistical analyses were carried out by SigmaStat software (Version 3.5; Chicago, IL). A $P$-value of $< 0.05$ was considered significant.

## RESULTS

### Experiment 1

Percent of progressive motility of spermatozoa was shown in Table 1. According to the result, the dose of 10 μM β-cyfluthrin was chosen for the second experiment.

### Experiment 2

***Motility of Spermatozoa*** There were no significant differences between the negative control and the control groups at 0, 24, and 48 time points (Table 2). While, motility of the samples exposed to β-cyfluthrin alone was significantly lower than the other groups at 24 and 48 h of storage ($P < 0.05$; Table 2). Co-supplementation of 10 and 25 mM *trans*-FA + β-cyfluthrin, improved motility of spermatozoa compared to β-cyfluthrin alone group at 24 and 48 h of storage ($P < 0.05$; Table 2). Simultaneous supplementation of semen samples with 50 mM of *trans*-FA+ β-cyfluthrin showed deleterious effect on the percentage of motile spermatozoa compared to control groups at 24 and 48 h of storage ($P < 0.05$; Table 2). Analysis over time revealed that, motility was lower in 24 and 48 h compared to 0 h in all experimental group ($P < 0.05$; Table 2).

**Table 2.** Percentage of forward progressive motility of rooster spermatozoa following exposure to β-cyfluthrin (Cyf; 10 μM) alone or concurrent with different amounts of *trans*-ferulic acid (FA; 10, 25, and 50 mM) and stored for various time points at 4°C.

| Treatment | Time of storage (h) | | |
|---|---|---|---|
| | 0 | 24 | 48 |
| Negative control | 68.00 ± 2.73 $^{A,a}$ | 41.75 ± 2.05 $^{AC,b}$ | 18.66 ± 1.45 $^{AC,c}$ |
| Control | 70.75 ± 2.28 $^{A,a}$ | 43.50 ± 1.84 $^{AC,b}$ | 21.66 ± 2.18 $^{AC,c}$ |
| β-cyf | 67.50 ± 2.72 $^{A,a}$ | 33.50 ± 1.89 $^{B,b}$ | 9.33 ± 1.20 $^{B,c}$ |
| β-cyf + 10 mM FA | 71.25 ± 2.65 $^{A,a}$ | 46.25 ± 2.42 $^{A,b}$ | 22.00 ± 2.00 $^{A,c}$ |
| β-cyf + 25 mM FA | 70.75 ± 2.32 $^{A,a}$ | 49.75 ± 2.75 $^{A,b}$ | 25.33 ± 2.18 $^{A,c}$ |
| β-cyf + 50 mM FA | 69.50 ± 1.75 $^{A,a}$ | 36.25 ± 1.65 $^{BC,b}$ | 13.66 ± 1.85 $^{BC,c}$ |

$^{A,B,C}$Values with different superscripts indicate difference ($P < 0.05$) among groups at each time point.

$^{a,b,c}$Values with different superscripts indicate significant differences ($P < 0.05$) between the data at the same raw.

**Table 3.** Percentage of viable spermatozoa following exposure to β-cyfluthrin (Cyf; 10 μM) alone or concurrent with different amounts of *trans*-ferulic acid (FA; 10, 25 and 50 mM) and stored for various time points at 4°C.

| Treatment | Time of storage (h) | | |
|---|---|---|---|
| | 0 | 24 | 48 |
| Negative control | 81.19 ± 3.44 $^{A,a}$ | 74.11 ± 1.64 $^{AB,ab}$ | 68.83 ± 0.87 $^{A,b}$ |
| Control | 79.56 ± 3.79 $^{A,a}$ | 74.86 ± 1.69 $^{AB,a}$ | 70.50 ± 1.17 $^{A,a}$ |
| β-cyf | 77.21 ± 3.04 $^{A,a}$ | 66.61 ± 1.92 $^{B,b}$ | 59.17 ± 1.68 $^{B,b}$ |
| β-cyf + 10 mM FA | 79.91 ± 2.69 $^{A,a}$ | 76.31 ± 2.01 $^{A,ab}$ | 72.15 ± 0.80 $^{A,b}$ |
| β-cyf + 25 mM FA | 80.33 ± 3.73 $^{A,a}$ | 78.96 ± 2.55 $^{A,a}$ | 74.10 ± 1.03 $^{A,a}$ |
| β-cyf + 50 mM FA | 80.80 ± 3.17 $^{A,a}$ | 74.52 ± 1.12 $^{AB,ab}$ | 71.30 ± 1.83 $^{A,b}$ |

$^{A,B}$Values with different superscripts indicate difference ($P < 0.05$) among groups at each time point.

$^{a,b}$Values with different superscripts indicate significant differences ($P < 0.05$) between the data at the same raw.

***Viability of Spermatozoa*** There were no significant differences in the percentage of viable spermatozoa among groups at time point 0 h ($P > 0.05$; Table 3). Exposure with β-cyfluthrin, decreased viability of spermatozoa at 24 h of storage, but this decrease was not statistically significant compared to control groups (Table 3). The percent of viable spermatozoa was less in β-cyfluthrin exposed group compared to other experimental group at 48 h of storage ($P < 0.05$; Table 3).

***MDA (μM/g Protein)*** Exposure of samples with 10 μM β-cyfluthrin alone caused significant increase in MDA values compared to the control groups at 24 and 48 h ($P < 0.05$; Figure 1). At these2 mentioned time points, supplementation of samples with 10 and 25 mM of *trans*-FA concurrent with β-cyfluthrin significantly decreased amounts of MDA compared to the β-cyfluthrin alone ($P < 0.05$; Figure 1). Moreover, MDA amounts were reported higher in 50 mM of *trans*-FA + β-cyfluthrin compared to controls and other combination groups at 48 h of storage ($P < 0.05$; Figure 1). Analysis over time revealed higher MDA values in β-cyfluthrin and β-cyfluthrin + 50 mM *trans*-FA groups at 48 h compared to 0 h ($P < 0.05$; Figure 1).

***TAC (mM/g Protein)*** Significant differences were not observed in amounts of TAC among experimental groups at 0 h of storage (Figure 1). At the next time point, the lowest TAC value was recorded in the β-cyfluthrin + 50mM *trans*-FA, which is significantly differed compared to negative control, control and β-cyfluthrin + 10 mM *trans*-FA ($P < 0.05$; Figure 2). At time point 48 h, TAC values of β-cyfluthrin and

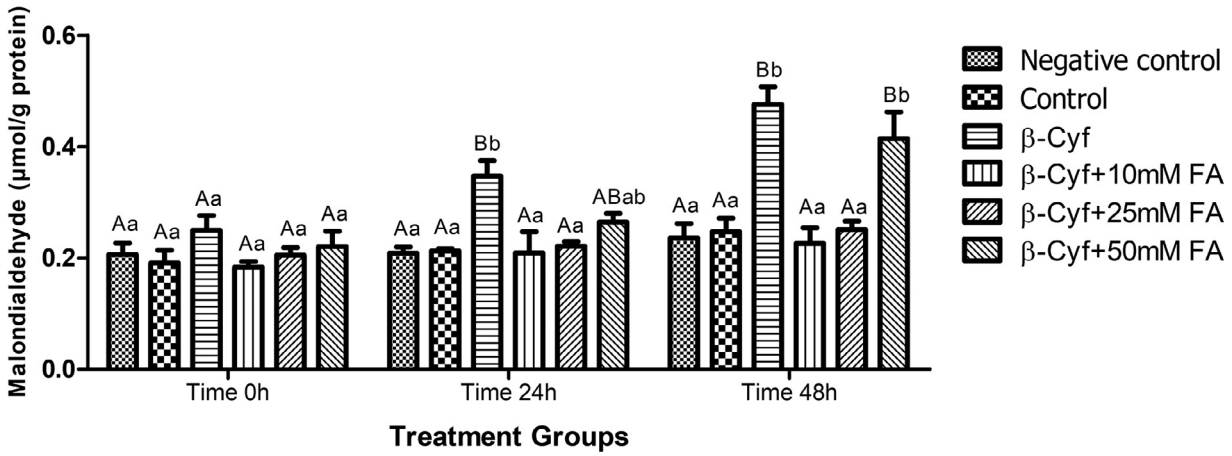


**Figure 1.** Amounts of malondialdehyde ($\mu$mol/g protein) in spermatozoa-diluent homogenate of rooster following exposure to $\beta$-cyfluthrin ($\beta$-Cyf; 10 $\mu$M) alone or concurrent with different amounts of *trans*-ferulic acid (FA; 10, 25, and 50 mM) and stored for various time points at 4°C. [A,B] Values with different letters indicate significant difference ($P < 0.05$) among groups at each time point. [a,b] Values with different letters indicate a difference ($P < 0.05$) over time within the experimental groups.

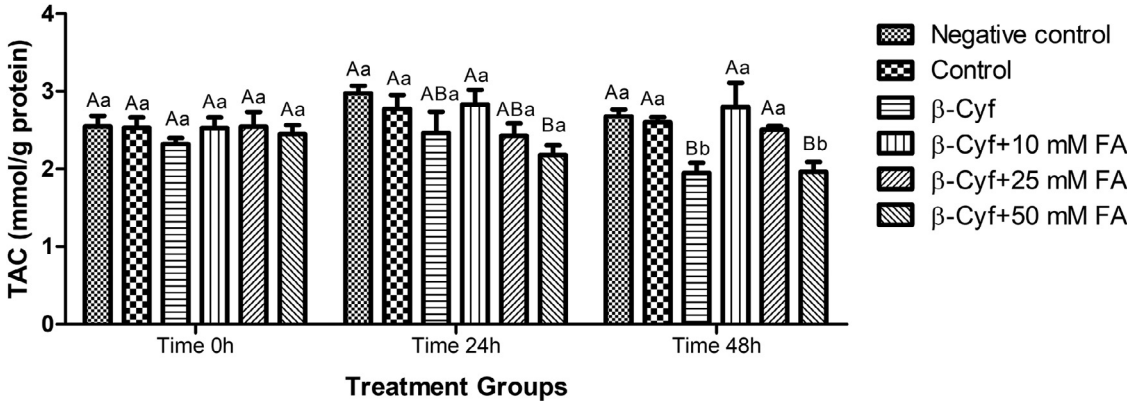


**Figure 2.** Total antioxidant capacity (TAC; mmol/g protein) in spermatozoa-diluent homogenate of rooster following exposure to $\beta$-cyfluthrin ($\beta$-Cyf; 10 $\mu$M) alone or concurrent with different amounts of *trans*-ferulic acid (FA; 10, 25, and 50 mM) and stored for various time points at 4°C. [A,B] Values with different letters indicate significant difference ($P < 0.05$) among groups at each time point. [a,b] Values with different letters indicate a difference ($P < 0.05$) over time within the experimental groups.

$\beta$-cyfluthrin + 50mM *trans*-FA groups were significantly lower compared to other experimental groups ($P < 0.05$; Figure 2). Moreover, $\beta$-cyfluthrin and $\beta$-cyfluthrin + 50 mM *trans*-FA groups showed lower amounts of TAC at time point 48 compared to time points 0 and 24 ($P < 0.05$; Figure 2).

***Total Nitrate-Nitrite ($\mu$M/g Protein)*** Values of nitrate-nitrite in the group exposed to $\beta$-cyfluthrin alone were significantly higher than other groups at 0, 24, and 48 h of storage ($P < 0.05$; Figure 3). Within group analysis did not show any significant variation among different time points of each treated group (Figure 3).

***Total HPO ($\mu$M/g Protein)*** The highest HPO values were observed in the samples exposed to $\beta$-cyfluthrin alone at 0 h of storage, which were significantly higher compared to other groups ($P < 0.05$; Figure 4). Significant differences were not detected among experimental groups at 24 and 48 time points (Figure 4). Within group analysis showed that HPO values of negative control, control and $\beta$-cyfluthrin (alone) groups were significantly differ between 0 and 48 h of storage ($P < 0.05$; Figure 4).

***SOD (U/mg Protein)*** Significant differences were not recorded in SOD activity among experimental groups at 0, 24, and 48 h of storage or within each experimental group among different time points (Figure 5).

## DISCUSSION

The first objective of the current study was to investigate the effect of different doses of $\beta$-cyfluthrin on rooster semen motility during cold storage up to 48 h. According to the results of first experiment, the mild toxic dose of $\beta$-cyfluthrin was chosen for the second experiment. Moreover, the second aim was to evaluate the probable protective role of *trans*-FA against $\beta$-cyfluthrin induced mild toxicity on different parameters of child stored rooster semen. It has been documented that some alterations and damages reduced fertility of spermatozoa but may not affect motility of spermatozoa (Chatterjee et al., 2001; Reddy et al., 2010). In other words, spermatozoa lose its fertility at first, and then the motility and other parameters (viability,

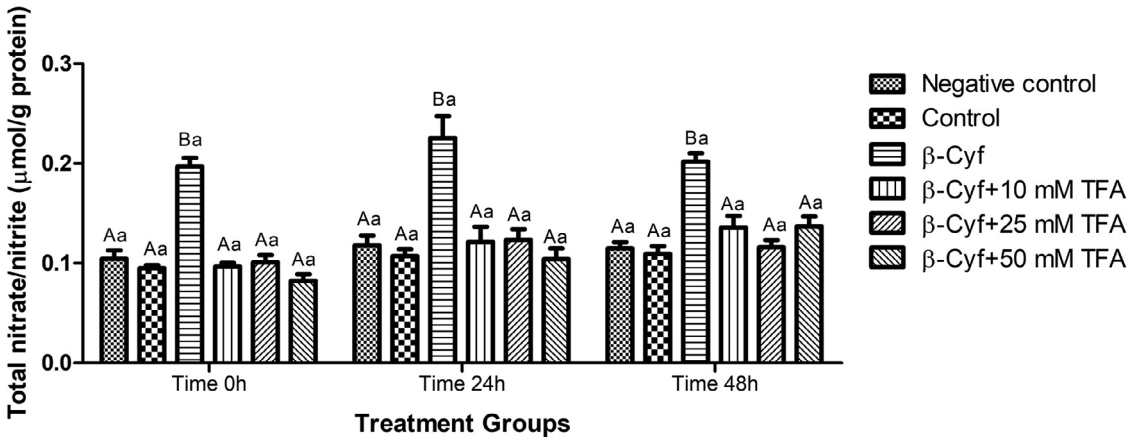


**Figure 3.** Total amounts of nitrate-nitrite ($\mu$mol/g protein) in spermatozoa-diluent homogenate of rooster following exposure to $\beta$-cyfluthrin ($\beta$-Cyf; 10 $\mu$M) alone or concurrent with different amounts of *trans*-ferulic acid (FA; 10, 25, and 50 mM) and stored for various time points at 4°C. [A,B] Values with different letters indicate significant difference ($P < 0.05$) among groups at each time point. No significant differences were observed within the experimental group among different time points.

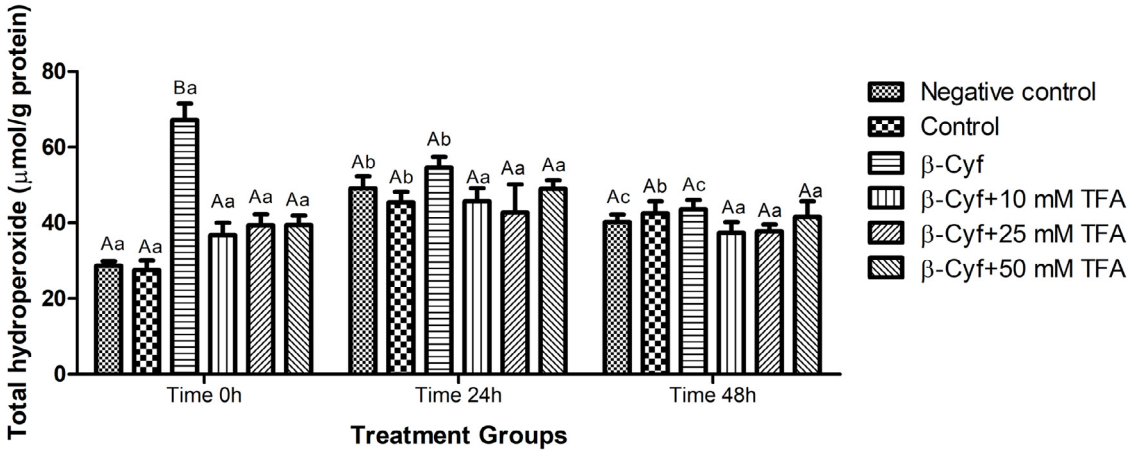


**Figure 4.** Total amounts of hydroperoxide ($\mu$mol/g protein) in spermatozoa-diluent homogenate of rooster following exposure to $\beta$-cyfluthrin ($\beta$-Cyf; 10 $\mu$M) alone or concurrent with different amounts of *trans*-ferulic acid (FA; 10, 25, and 50 mM) and stored for various time points at 4°C. [A,B] Values with different letters indicate significant difference ($P < 0.05$) among groups at each time point. [a,b,c] Values with different letters indicate a difference ($P < 0.05$) over time within the experimental groups.

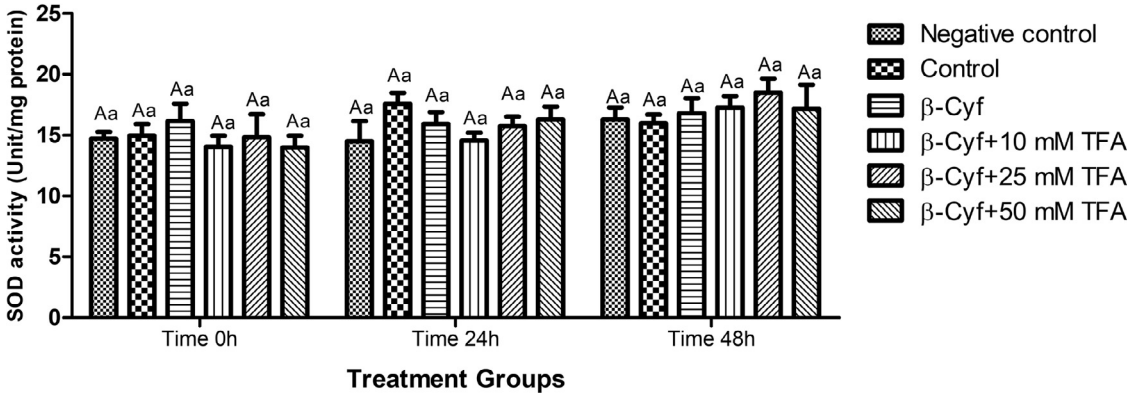


**Figure 5.** Superoxide dismutase activities (unit/mg protein) in spermatozoa-diluent homogenate of rooster following exposure to $\beta$-cyfluthrin ($\beta$-Cyf; 10 $\mu$M) alone or concurrent with different amounts of *trans*-ferulic acid (FA; 10, 25, and 50 mM) and stored for various time points at 4°C. No significant differences were observed among groups or within a group among different time points.

functionality of spermatozoa membrane) would be affected, especially during storage as liquid at refrigerator temperature. Accordingly, in the first experiment, motility of spermatozoa (as one of the main factors of spermatozoa) was chosen to be evaluated. A few studies are available on the effects of cyfluthrin toxicant on the quality of animal semen. The results of the current study indicated that different concentrations of $\beta$-cyfluthrin

had negative effects on the progressive motility of in vitro stored rooster spermatozoa. Moreover, β-cyfluthrin at 10 μM levels affected different parameters of rooster semen over cold preservation at 4°C. in this regard, the relationship between urinary metabolites of pyrethroid and the parameters related to human semen was evaluated (Meeker et al., 2008). They reported the negative correlation between amounts of urinary metabolites of pyrethroid insecticides and the quality of semen and DNA damage of spermatozoa. While, other report cited that cyfluthrin and pyrethrin had nonsignificant effects on semen parameters and serum testosterone concentrations of studied bulls over 18 wk (Stewart et al., 2016). Their findings were in consistent with the results of French et al. (2014). However, results of another study revealed that these compounds did not reduce sperm motility, but lowered serum concentrations of testosterone in the bulls at wk 9 (Stewart et al., 2015).

Several characteristics of *trans*-FA in living organisms have been investigated. Many researchers have focused on its antioxidant properties (Masuda et al., 2006; Fong et al., 2016). There are few researches available in the literature about the effects of *trans*-FA on the genital system of animals (Zheng and Zhang, 1997). According to the authors' knowledge, no study has been conducted on the effects of *trans*-FA on rooster semen parameters at cold storage state. The results of the second experiment of the study confirmed that *trans*-FA had the potential of sperm protection against the deleterious effects of mild toxic doses of β-cyfluthrin. A research on diabetic rats revealed that treatment with FA, restored and improved spermatozoa count, motility, and viability (Roy et al., 2014). Improvement in progressive motility and viability of rooster spermatozoa could attributed to protective effects of *trans*-FA upon semen cold storage. The most protective effects of *trans*-FA were observed at 10 and 25 mM levels.

Due to high amounts of polyunsaturated fatty acids in the spermatozoa membrane and their low antioxidant capacity, spermatozoa are very sensitive to endogenous produced ROS species during cold storage or added exogenous toxic substances which mimic oxidative toxicity process (Alvarez and Storey, 1995; Holt, 2000). Irreversible damages and changes in the function and fertilization potential of spermatozoa has been reported during cooling process of semen sample (Yeste, 2016). Enrichment of semen extender have been proposed to ameliorate oxidative toxicity during semen storage as liquid or freeze state (Câmara et al., 2011; Eslami et al., 2016; Benhenia et al., 2018; Ghaniei et al., 2019). Results of the current study revealed the higher amounts of MDA in β-cyfluthrin alone exposed group compared to β-cyfluthrin plus 10 and 25 mM *trans*-FA treated groups. Moreover, total amounts of antioxidant were higher in 10 and 25 mM *trans*-FA groups compared to β-cyfluthrin alone exposed group. However, SOD activity (an antioxidant enzyme) did not differ among treated groups. In this regard, rooster semen enrichment with ellagic acid and lycopene could not change SOD activity, but improved antioxidant capacity and glutathione peroxidase levels (Najafi et al., 2018, 2019). Accordingly, it was proposed that measurement of glutathione peroxidase is more indicative than SOD activity during in vitro semen processing studies (Mortazavi et al., 2020). It seems that peroxidative reaction is one of the pathways of induced toxicity by pyrethroid insecticides over in vitro exposure (Romero et al., 2012; Park et al., 2017). Moreover, nitrosative stress (as shown by elevation of total nitrate-nitrite) has been confirmed during exposure of rooster semen by β-cyfluthrin in the present study. Broad physiological activity of FA, such as antioxidative, antiallergic, anticarcinogenic, hepatoprotective, antimicrobial, metal chelative, and anti-inflammatory have been reported (Li et al., 2012; Kumar and Pruthi, 2014). In this regard, a recent study revealed that exogenous added FA could protected mitochondria, acrosome, and plasma membrane integrity of stallion spermatozoa over 8 h storage at 4°C (Affonso et al., 2017). Another study indicated that different amounts of exogenous added FA improved motility and viability of spermatozoa and reduced the lipid peroxidation levels in both samples of fertile and infertile men (Zheng and Zhang, 1997). The negative correlation between levels of MDA and quality of fresh collected or stored semen samples was confirmed by researches (Lewis et al., 1995; Bucak et al., 2010). Another study indicated protective roles of FA via the reduction of lipid peroxidation levels and enhancing enzymatic antioxidant activities in diabetic treated rats (Roy et al., 2014). However, results of the previous study revealed the negative effects of FA on copulatory behavior and hatchability of eggs following oral supplementation to quails (de Man and Peeke, 1982). The inconsistency about FA effects among biological studies might be due to different test conditions, target species, and doses of the used FA. According to circumstances of the current study, it seems that protective role of FA may due to attenuation of peroxidative and nitrosative toxicity induced by β-cyfluthrin over preservation of liquid semen at 4°C. Moreover, the other probable protective mechanism of FA is the elevation of antioxidant capacity after exogenous addition to rooster semen diluent.

Interestingly, rooster semen supplementation with 50 mM trans-FA, not only did not provide adequate protection, but also showed deleterious effects on motility of spermatozoa and the measured metabolites within the spermatozoa-diluent homogenate, as shown by MDA elevation and TAC reduction levels. This finding is consistent with the results of previous studies that noted high concentrations of phenolic compounds act like a pro-oxidant, produced free radicals, stimulated oxidative toxicity, and induced nitrosylation of proteins (Jiang et al., 2000; Du et al., 2017).

To the best of our knowledge, no study has been done to evaluate simultaneous effects of β-cyfluthrin and *trans*-FA on quality of stored rooster spermatozoa. In this study, the safeguarding effects of *trans*-FA in 2 concentrations of 10 and 25 mM were shown on the rooster semen samples exposed to mild toxic doses of β-cyfluthrin. The results of this study could suggest *trans*-FA

as an additive in the preservation of rooster semen during in vitro storage. However, the study of varying doses of *trans*-FA on semen samples of different animal species during extended period of liquid storage and cryostorage state were suggested by the authors. Moreover, studies are required to evaluate the response of in vivo challenge of $\beta$-cyfluthrin alone and in combination with *trans*-FA on different parameters of spermatozoa, fertility, and oxidative/antioxidative balance in roosters.

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## DISCLOSURES

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper. The authors have no conflicts of interest.

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