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Evaluation of Antioxidant Enzymes Activity, Lipid Peroxidation and Sperm Quality in Broiler Breeder Roosters Fed Whey Protein and Sodium Selenite

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Abstract

The objective of this study was to investigate the effect of whey protein concentrate (WPC) and selenium (Se) supplementation on sperm quality, antioxidant enzymes activity and lipid peroxidation in seminal plasma, liver and testis of roosters. Forty-five Ross-308 broiler breeder roosters aged 60 weeks were used for an eight-week period in a 3 × 3 factorial arrangement of dietary treatments. Three levels of WPC (0.0, 1.5 and 3.0% of diet) and selenium supplementation (0.0, 0.2 and 0.4 mg/kg of diet) with five replications were tested. Total and progressive sperm motility, sperm concentration, plasma membrane integrity, and sperm viability were significantly lower in birds treated with Se supplementation-free diet ($P < 0.05$). Also, abnormal sperms were significantly higher in Se supplementation-free diets when compared to the diets supplemented with 0.4 mg/kg Se ($P < 0.05$). The use of 1.5% of WPC resulted in significantly increased total and progressive sperm motility compared to the WPC-free diet ($P < 0.05$). Selenium at the level of 0.4 mg/kg along with 3.0% WPC were associated with significantly increased Glutathione peroxidase and superoxide dismutase activity in seminal plasma as compared to other levels ($P < 0.05$). The highest level of total antioxidant capacity (TAC) in seminal plasma was observed at the level of 0.2 mg Se ($P < 0.05$). Further, 3.0% WPC resulted in significantly increased TAC concentration in seminal plasma compared to the WPC-free diet ($P < 0.05$). Moreover, the Malondialdehyde (MDA) level of seminal plasma in selenium supplementation-free diets was significantly higher than those of other levels ($P < 0.05$). Glutathione peroxidase activity, TAC, and MDA levels in the testis and liver were not affected by the WPC and Se levels. It can be concluded that dietary inclusion of WPC and Se improved the semen quality, increased antioxidant enzymes activity and decreased lipid peroxidation in seminal plasma of broiler breeder roosters.

Introduction

Rooster spermatozoa are characterized by very high proportions of long-chain polyunsaturated fatty acids (PUFAs), mainly arachidonic (20:4n-6) and docosatetraenoic (22:4n-6) fatty acids (Kelso *et al*., 1996; Cerolini *et al*., 1997; Surai *et al*., 1998). In the presence of reactive oxygen species (ROS), these high levels of PUFAs can easily undergo lipid peroxidation (LPO) (Partyka *et al*., 2012). Reactive oxygen species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^\bullet$) are reactive molecules generated during oxygen reduction. Peroxidation of PUFAs in sperm cell membranes is a self-propagating, autocatalytic reaction increasing cell dysfunction related to the loss of membrane function and integrity (Sanocka and Kurpisz, 2004). Thus, the function and viability of the spermatozoa are affected by creating higher concentrations of these molecules (John Aitken *et al*., 1989; Birben *et al*., 2012). Although the sperm total antioxidant capacity (TAC) in the cytoplasm is low, enzymatic antioxidant compounds in the seminal

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plasma comprising superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and also natural antioxidants such as vitamins E, A, C, uric acid, glutathione and carotenoids function as a defense mechanism against LPO and ROS (Bréque *et al*., 2003; Cerolini *et al*., 2006). Superoxide dismutase is a key element for preliminary antioxidant defense in semen (Surai *et al*., 2001). It catalyzes the superoxide anion (O_2^-) dismutation to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), which is then eliminated by CAT and GPx (Lamirande and Gagnon, 1993). Glutathione peroxidase is a seleno-cysteine containing tetrameric enzyme, which reduces lipid peroxides and H_2O_2 to lipid alcohols and water, respectively (Fanucchi, 2004; Tabet and Touyz, 2007). The existence of GPx and SOD in the seminal plasma and spermatozoa of chickens, geese, turkeys, ducks and guinea fowls was demonstrated by Surai *et al*. (1998) .

Selenium (Se) is an essential trace element playing an important role in male fertility (Hansen and Deguchi, 1996; Surai, 2002). Selenium exists in the body as a part of at least 25 selenoproteins involved in regulating different physiological functions such as redox regulation of gene expression, antioxidant protection, sperm structure integrity, and thyroid metabolism (Surai, 2002; Kryukov *et al*., 2003). Selenium, as a component of spermatozoa, is an essential element for spermatogenesis (Wu *et al*., 1973). The deficiency in dietary Se can lead to the decreased numbers of normal spermatozoa per ejaculate, reduced motility and fertilizing capacity in birds and mammals (Surai, 2000). Selenium also has antioxidative features through inclusion in the GPx's active site (Hansen and Deguchi, 1996).

Whey is a by-product of cheese manufacturing. Since it has high nutritional value, it can be utilized as a feed additive in feeding poultry (Rastad *et al*., 2008). The whey protein (WP) has high biological value due to considerable concentrations of the essential amino acids, which can quickly elevate the amino acids content of plasma (Hayes and Cribb, 2008). It is suggested that the presence of β-lactoglobulin, serum albumin, α-lactalbumin, and immunoglobulins in whey protein could boost the immune system and also prolong animal survival (De Wit, 1998). Further, whey proteins possess antioxidant features since they contain glutamylcysteine groups in their structures (Bounous and Gold, 1991; Lands *et al*., 1999). It was found that cysteine-rich whey protein concentrate (WPC) enhances intracellular glutathione levels (Bounous, 2000; Micke *et al*., 2001). Moreover, dietary supplementation of glutathione precursor, cysteine, improves the immune response in animals fed WPC (Bounous *et al*., 1989).

Therefore, the aim of this study was to evaluate the effect of feeding WPC and selenium supplon sperm quality, antioxidant enzyme activity and lipid peroxidation intensity in seminal plasma, liver and testis of broiler breeder roosters.

Materials and methods

Ethical issue

All experimental protocols in the present study were approved by the Animal Care and Use Committee of Ferdowsi University of Mashhad and performed following guidelines and regulations to minimize pain, suffering and distress to birds.

Animals, diets and experimental design

Forty-five Ross-308 broiler breeder roosters aged 60 weeks were kept in individual cages under controlled environment (20-23°C, 15L: 9D light: dark schedule). Before starting the experiment, the roosters were adapted to the experimental conditions and abdominal semen collections for two weeks.

The present study was performed based on a completely randomized design in a 3 × 3 factorial arrangement with five replications in each treatment. Roosters were fed with dietary treatments containing three levels of WPC (0.0, 1.5, and 3.0% of diet) and sodium selenite (0.0, 0.2, and 0.4 mg/kg of diet) for 8 weeks. Nutrient requirements and feed intake were based on the Ross-308 parent stock manual instructions. All diets were balanced to be isonitrogenous and isoenergetic. Water was freely accessible to the roosters. Experimental diets were formulated to meet the nutritional recommendations of broiler breeder roosters by UFFDA software (Table 1).

Whey protein concentrate was given by Karen Pharma Company whereit was prepared based on the brochure, from sweet and fresh dairy whey processed by a special cross-flow filtration, agglomeration and surface treatment. Proper methods were used to analyze WPC (Table 1). The Kjeldahl process was used to measure crude protein (CP: N×6.25). The analytical technique flowchart for acidic and neutral sample hydrolysis was based on AOAC Official Process (AOAC, 2000). After sample oxidation with amino acids and performic acid through an automatic amino acids analyzer, hydrolysis was performed for sulphur amino acids, and the values were utilized as a reference for the development of Near Infrared Reflectance calibration models. An NRC table was used To calculate energy content, (NRC, 1994). Using a spectrophotometer (Shimadzu 6300 AAS AA/AE) phosphorus, calcium, and sodium content was measured. Fehling's reagent was applied to calculate lactose amount .

Table 1. Ingredients and nutrient composition of experimental diets fed to broiler breeder roosters

Ingredients (%)	WPC 0 (Without WPC)	WPC 1.5 %	WPC 3.0%
Corn	63.79	62.89	60.49
Soybean Meal	6.76	3.74	0.50
Wheat bran	24.21	26.68	30.85
WPC[1]	0.00	1.50	3.00
Dicalcium phosphate	1.32	1.31	1.29
$CaCo_3$	0.93	0.92	0.93
Vegetable oil	2.00	2.00	2.00
Salt	0.31	0.26	0.25
$NaHCO_3$	0.07	0.10	0.10
Mineral Premix[2]	0.25	0.25	0.25
Vitamin Premix[3]	0.25	0.25	0.25
DL-Methionine	0.11	0.1	0.09
Nutrient level (%)			
Metabolizable Energy (kcal/kg)	2790	2790	2790
Crude protein	11.93	11.93	11.93
Linoleic Acid	2.88	2.93	3.01
Calcium	0.70	0.70	0.70
Available phosphorus	0.35	0.35	0.35
Sodium	0.18	0.18	0.18
Potassium	0.62	0.60	0.60
Chloride	0.23	0.20	0.19
Fiber	4.54	4.59	4.79
Methionine	0.31	0.31	0.31
Methionine + Cystine	0.55	0.57	0.58
Lysine	0.49	0.53	0.56
Threonine	0.42	0.46	0.50
Arginine	0.71	0.66	0.62
Tryptophan	0.13	0.12	0.10
Valine	0.56	0.58	0.59

Abbreviations: CP, crude protein; ME, metabolisable energy; WPC, whey protein concentrate.

[1] Whey Protein Concentrate (WPC) contains: CP, 76.26%; moisture, 5.60%; dry matter, 93.90%; methionine, 1.61%; cysteine; 1.97%; methionine+Cystine, 3.58%; lysine, 7.20%; threonine, 5.59%; arginine, 2.06%; isoleucine, 4.94%; leucine, 8.45%; valine, 4.67%; histidine, 1.58%; phenyl alanine, 2.80%; glycine, 1.55%; serine, 4.31%; proline, 4.36%; alanine, 3.97%, aspartic, 8.90%; glutamic, 13.73%; calcium, 0.67%; phosphorous, 0.30%; sodium, 0.24%; ether extract, 0.80%, ash, 2.60%; lactose; 9.1%.

[2] Mineral Premix (per kilogram of diet): selenium, Includes 3 levels (0.0, 0.2 and 0.4 mg Se/kg of diet); copper, 10 mg; iron, 50 mg; iodine, 2 mg; manganese, 120 mg; zinc, 110 mg.

[3] Vitamin Premix (per kilogram of diet): vitamin A, 11,000 IU; vitamin D3, 3500 IU; vitamin E, 100 IU; vitamin K, 5 mg; thiamine, 3 mg; riboflavin, 12 mg; niacin, 55 mg; biotin, 0.25 mg; pyridoxine, 4 mg; vitamin B12, 0.03 mg; folic acid, 2 mg; pantothenic acid, 15 mg.

Semen assessments

Semen samples were collected and evaluated from each rooster every two weeks by the abdominal massage method (Burrows and Quinn, 1937). Seminal volume was measured in graduated collecting tubes and only clean ejaculates were utilized. Computer-assisted sperm analysis (HFT CASA V6.50, Hooshmand Fanavar Tehran Co., Iran) was used to evaluate the sperm motility characteristics. Briefly, the semen was diluted (1:200 in 0.9% NaCl), then 10 µl of diluted semen was placed on the pre-warmed slide, covered with a coverslip and studied using a phase-contrast microscope (Labomed, Lx 400, USA; magnification: X 400). Total and progressive sperm motility was stated as a percentage of spermatozoa representing moderate to rapid movement progressively (Akhlaghi *et al*., 2014). Using eosin-nigrosin staining by light microscope (Labomed, Lx 400, USA), sperm abnormality and viability percentages were assessed (200 spermatozoa/slide). Sperm concentration was measured using a hemocytometer (Smith and Mayer, 1955).

Plasma membrane integrity

Hypo-osmotic swelling (HOS) test was done to assess sperm plasma membrane integrity (Santiago-Moreno

et al., 2009). Briefly, 50 µL was added toHOS solution and incubated at 37˚C for 30 min. Preparing a specimen on a slip-covered slide, the HOS positive spermatozoa percentage was determined via phase-contrast microscope (magnification: X 1000; Labomed, Lx 400, USA).

Biochemical assays

The semen samples (300 µL each) were centrifuged at 4000×*g* for 10 min (Asa tajhiz pars, RST, Iran), supernatants were removed and stored at −70˚C until further analysis. Finally, after killing the animals, the liver and testis were quickly removed and washed with normal saline to eliminate residual blood. They were then kept at −70˚C to analyze the antioxidant status and lipid peroxidation susceptibility.

Lipid peroxidation

Malondialdehyde (MDA) concentration as an index of the lipid peroxidation in the liver, testis and seminal plasma samples was measured using the Thiobarbituric acid (TBA) reaction (Chuaychu-Noo *et al*., 2017). Briefly, placing 20 µL of the specimen in a glass centrifuge tube, 1/12 N H_2SO_4 (4.0 mL) was added and gently mixed. Then, it was mixed by adding 0.5 mL of 10% phosphotungstic acid. The mixture was then allowed to rest for 5 min at room temperature and centrifuged for 10 min at 1600×*g*. By discarding the supernatant, the sediment was mixed with 2.0 mL of 1/12 N H_2SO_4 followed by 10% phosphotungstic acid (0.3 mL). After centrifuging the mixture for 10 min at 1600×*g*, the sediment was suspended in 1.0 mL of distilled water. Then, 1.0 mL of 0.67% TBA reagent (w/v) was addedthe reaction mixture was heated at 95°C for 60 min,. Then, adding 3.0 mL of n-butanol after cooling with tap water, the mixture was vigorously shaken. The specimens were centrifuged 3000×*g* for 10 min to achieve a clear supernatant. UV-Visible spectrophotometer was used to determine the MDA in the sample, at 532 nm. The MDA standard was used to prepare the standard curve. The samples' MDA concentration was stated as nM and µM (Yagi, 1976).

Glutathione peroxidase activity

Glutathione peroxidase activity was determined using Randox as described by Paglia and Valentine (1967). The sample was mixed with t-butyl hydroperoxide in a ratio of 4:1 as well as a solution of distilled water comprising buffer (0.025 M Na_2EDTA and 0.25 M KH_2PO_4), 10 mg glutathione (GSH), NADPH, GSH reductase, and 940 mL Kbuffer. The GPx activity was then measured at intervals of 10 s for 60 s by recording the light absorption rate.

Superoxide dismutase activity

Determining SOD activity was based on Sun *et al.*'s study (1988) utilizing Randox. This technique is oriented by the inhibition of nitroblue tetrazolium (NBT) reduction by xanthine-xanthine oxidase utilized as a superoxide generator. One SOD activity was stated as the quantity of enzyme causing inhibition of 50% for the NBT reduction rate. SOD activity was considered as a unit for mg/protein (Paoletti *et al*., 1986).

Total antioxidants capacity

To measure TAC, a Randox total antioxidant status kit was used. In this kit, by incubation of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) with H_2O_2 and peroxidase, the radical cation $ABTS^+$ was produced. It had a stable blue-green color, which was measured at 600 nm (Miller *et al*., 1993).

Statistical analysis

To analyze the data, the GLM procedure of SAS software version 9.1 (SAS, 2002) was used and mean differences if significant ($P < 0.05$) were compared by Tukey's multiple range test. The time effect, main effects, and interactions between WPC and Se levels were studied on the data.

Results

The results of semen evaluations are presented in Table 2. Different levels of WPC and Se affect total and progressive sperm motility. Selenium at the levels of 0.2 and 0.4 mg/kg, significantly improved the total and progressive sperm motility compared to selenium-free diet ($P < 0.05$). Also, 1.5% WPC resulted in significantly increased total and progressive sperm motility compared to the WPC-free diet ($P < 0.05$). The interaction effect of WPC × Se did not affect the seminal volume and sperm concentration. Though, the sperm concentration was affected by Se alone. Sperm concentration at the level of 0.4 mg Se was significantly higher than that of the selenium-free diet ($P < 0.05$). Sperm membrane integrity was significantly affected by experimental diets. Although Se at the levels of 0.2 and 0.4 mg significantly increased membrane integrity of sperm compared to selenium-free diet ($P < 0.05$), the effect of WPC on this trait was not significant. The effect of experimental diets on sperm viability was not significant, but Se alone could affect sperm viability. Live sperm at the level of 0.4 mg Se was significantly higher than the selenium supplementation-free diet ($P< 0.05$). The diets without Se supplementation showed higher percentage of abnormal sperm than diets supplemented with 0.4 mg/kg of Se ($P < 0.05$).

Table 2. The effect of different levels of whey protein concentrate (WPC) and selenium (Se) on semen quality[1] (LSmean ± SE)

WPC (%)	Se (mg/Kg)	Total sperm motility (%)	Progressive sperm motility (%)	Seminal volume (mL/ rooster)	Sperm concentration ($\times 10^9$ cells/mL)	HOS[2] (%)	Live sperm (%)	Abnormal sperm (%)
0.0	0.0	86.07± 1.47^{bc}	70.2 ±1.41 ^{bcd}	0.41± 0.04	3.02 ± 0.23	79.27± 1.72^{cd}	95.07± 0.63	6.27±0.67^{ab}
0.0	0.2	86.60± 1.27^{bc}	71.27±1.30 ^{bcd}	0.37± 0.03	2.92 ± 0.15	80.53±1.31^{bcd}	95.60± 0.84	6.07±1.23^{ab}
0.0	0.4	87.80±1.06^{abc}	72.27±1.00 ^{abcd}	0.42± 0.02	3.17 ± 0.15	82.47±1.50^{abc}	95.80± 0.57	5.60± 0.65^{b}
1.5	0.0	83.60± 1.35 ^{c}	68.53±1.30 ^{cd}	0.39± 0.03	2.94 ± 0.25	77.47± 1.59^{cd}	92.80± 1.55	6.93±0.72^{ab}
1.5	0.2	91.20± 0.79^{ab}	75.73±0.92 ^{ab}	0.41± 0.04	3.17 ± 0.17	84.27±1.62^{abc}	95.27± 0.60	6.00±0.41^{ab}
1.5	0.4	93.07± 1.08 ^{a}	77.87±1.10 ^{a}	0.42± 0.05	3.45 ± 0.18	81.20±1.52^{bcd}	95.60± 0.97	5.27±0.53 ^{b}
3.0	0.0	83.60± 1.80 ^{c}	67.13±1.94 ^{d}	0.40± 0.02	2.91 ± 0.23	75.27±1.61^{d}	92.1 ± 1.46	9.87±1.43 ^{a}
3.0	0.2	89.60± 1.03^{ab}	74.07±0.98 ^{abc}	0.43± 0.03	3.31 ± 0.17	86.53±1.55^{ab}	94.47± 0.94	6.67±0.89^{ab}
3.0	0.4	92.40± 1.09 ^{a}	77.27±1.24 ^{a}	0.44± 0.04	3.26 ± 0.13	88.80± 1.51^{a}	95.07± 0.71	5.73±0.85 ^{b}
Main effects								
WPC (%)								
0.0		86.82± 0.72 ^{b}	71.24±0.72 ^{b}	0.40±0.02	3.03 ± 0.10	80.75 ± 0.88	95.49 ± 0.39	5.98 ± 0.50
1.5		89.28± 0.87 ^{a}	74.04±0.87 ^{a}	0.41±0.02	3.19 ± 0.12	80.97 ± 0.98	94.56 ± 0.65	6.07 ± 0.34
3.0		88.53±0.94 ^{ab}	72.82±1.03 ^{ab}	0.43±0.02	3.16 ± 0.10	83.53 ± 1.25	93.89 ± 0.64	7.42 ± 0.67
Se (mg/kg)								
0.0		84.42± 0.89 ^{b}	68.62±0.91^{b}	0.40±0.02	2.96±0.13 ^{b}	77.33± 0.96 ^{b}	93.33± 0.75 ^{b}	7.69±0.61 ^{a}
0.2		89.13± 0.66 ^{a}	73.69±0.67^{a}	0.40±0.02	3.13±0.09 ^{ab}	83.77± 0.92 ^{a}	95.11± 0.46^{ab}	6.24±0.51^{ab}
0.4		91.09± 0.70 ^{a}	75.80±0.74^{a}	0.43±0.02	3.29±0.09 ^{a}	84.15± 0.99 ^{a}	95.49± 0.44 ^{a}	5.53±0.39 ^{b}
P-values								
WPC		0.0431	0.0236	0.7121	0.576	0.0546	0.135	0.080
Se		<.0001	<.0001	0.5899	0.014	<.0001	0.018	0.010
WPC × Se		0.0073	0.0086	0.9138	0.655	0.0014	0.752	0.029
Time		0.0490	0.0813	0.8416	0.625	0.3626	0.319	0.344
Diet[3] × Time		0.5386	0.2766	0.9999	0.998	0.4742	0.8740	0.691

[1] 45 roosters (five roosters per each group) fed diet containing 3 levels of WPC (0.0, 1.5 and 3.0% of diet) and 3 levels of selenium supplement (0.0, 0.2 and 0.4 mg Se/kg of diet) for 8 weeks (62-70 weeks of age).
[2] The percentage of sperm with swollen tails in a hypo-osmotic (HOS) solution (100 mOsm/Kg).
[3] Diet: WPC × Se
[a-d] Within each column, values with different superscript(s) differ significantly ($P < 0.05$).

Table 3. The effect of different levels of whey protein concentrate (WPC) and selenium (Se) on seminal plasma antioxidant enzymes activity and lipid peroxidation[1] (LSmean ± SE)

WPC (%)	Se (mg/Kg)	Glutathione peroxidase (U/mL)	Superoxide dismutase (U/mL)	Total antioxidants capacity (mM/L)	Malondialdehyde (μM/mL)
0.0	0.0	7.46 ± 0.65 ^{c}	58.20 ± 1.74 ^{c}	5.99 ± 0.30 ^{bc}	1.46 ± 0.04 ^{bc}
0.0	0.2	7.57 ± 0.46 ^{c}	59.87 ± 1.90 ^{bc}	7.54 ± 0.35 ^{ab}	1.47 ± 0.03 ^{abc}
0.0	0.4	8.83 ± 0.47 ^{bc}	58.13 ± 1.37 ^{c}	7.38 ± 0.49 ^{ab}	1.29 ± 0.03 ^{d}
1.5	0.0	9.00 ± 0.49 ^{ab}	60.93 ± 1.07 ^{bc}	4.87 ± 0.19 ^{c}	1.61 ± 0.03 ^{a}
1.5	0.2	7.92 ± 0.43 ^{bc}	62.40 ± 0.95 ^{bc}	8.82 ± 0.34 ^{a}	1.40 ± 0.02 ^{bcd}
1.5	0.4	7.45 ± 0.45 ^{c}	65.80 ± 1.47 ^{ab}	7.61 ± 0.64 ^{ab}	1.33 ± 0.03 ^{cd}
3.0	0.0	8.52 ± 0.38 ^{bc}	63.93 ± 1.95 ^{abc}	7.66 ± 0.23 ^{ab}	1.49 ± 0.04 ^{ab}
3.0	0.2	7.86 ± 0.56 ^{bc}	66.30 ± 1.39 ^{ab}	8.88 ± 0.42 ^{a}	1.42 ± 0.04 ^{bcd}
3.0	0.4	10.38 ± 0.37 ^{a}	69.63 ± 1.71 ^{a}	6.66 ± 0.63 ^{b}	1.33 ± 0.03 ^{cd}
Main effects					
WPC (%)					
0.0		7.95 ± 0.32 ^{b}	58.73 ± 0.96 ^{c}	6.97 ± 0.25 ^{b}	1.40 ± 0.02
1.5		8.13 ± 0.28 ^{b}	63.04 ± 0.74 ^{b}	7.09 ± 0.35 ^{ab}	1.45 ± 0.02
3.0		8.92 ± 0.30 ^{a}	66.62 ± 1.02 ^{a}	7.73 ± 0.29 ^{a}	1.42 ± 0.02
Se (mg/kg)					
0.0		8.33 ± 0.31 ^{ab}	61.02 ± 0.99 ^{b}	6.17 ± 0.22 ^{c}	1.52 ± 0.02 ^{a}
0.2		7.78 ± 0.27 ^{b}	62.85 ± 0.92 ^{ab}	8.42 ± 0.23 ^{a}	1.43 ± 0.02 ^{b}
0.4		8.89 ± 0.30 ^{a}	64.52 ± 1.21 ^{a}	7.21 ± 0.34 ^{b}	1.32 ± 0.02 ^{c}
P-values					
WPC		0.0005	<.0001	0.0325	0.3080
Se		0.0002	0.0175	<.0001	<.0001
WPC × Se		<.0001	0.2341	<.0001	0.0191
Time		<.0001	0.0082	<.0001	0.3843
Diet[2] × Time		0.0011	0.1114	0.1402	0.0008

[1] 45 roosters (five roosters per each group) fed diet containing 3 WPC levels (0.0, 1.5 and 3.0% of diet) and 3 levels of selenium supplement (0.0, 0.2 and 0.4 mg Se/kg of diet) for 8 weeks (62-70 weeks of age).
[2] Diet: WPC × Se
[a-d] Within each column, values with different superscript(s) differ significantly ($P < 0.05$).

Table 4. The effect of different levels of whey protein concentrate (WPC) and selenium (Se) on testis antioxidant enzymes activity and lipid peroxidation[1] (LSmean ± SE)

WPC (%)	Se (mg/Kg)	Glutathione peroxidase (U/mg protein)	Superoxide dismutase (U/mg protein)	Total antioxidans capacity (mmol/L)	Malondialdehyde (nmol/mg protein)
0.0	0.0	1.17 ± 0.02	9.88 ± 0.20 [a]	0.17 ± 0.02	0.17 ± 0.01 [ab]
0.0	0.2	1.15 ± 0.02	9.29 ± 0.21 [ab]	0.17 ± 0.01	0.14 ± 0.01 [ab]
0.0	0.4	1.21 ± 0.13	8.79 ± 0.22 [ab]	0.19 ± 0.02	0.12 ± 0.004 [b]
1.5	0.0	1.05 ± 0.05	8.67 ± 0.31 [b]	0.17 ± 0.01	0.14 ± 0.01 [ab]
1.5	0.2	1.21 ± 0.05	9.35 ± 0.29 [ab]	0.18 ± 0.02	0.15 ± 0.01 [ab]
1.5	0.4	1.21 ± 0.04	9.73 ± 0.08 [ab]	0.16 ± 0.02	0.18 ± 0.02 [ab]
3.0	0.0	1.22 ± 0.03	9.61 ± 0.39 [ab]	0.16 ± 0.03	0.19 ± 0.02 [a]
3.0	0.2	1.17 ± 0.01	9.11 ± 0.20 [ab]	0.16 ± 0.01	0.16 ± 0.01 [ab]
3.0	0.4	1.13 ± 0.03	8.91 ± 0.19 [ab]	0.13 ± 0.02	0.15 ± 0.01 [ab]
Main effects					
WPC (%)					
0.0		1.18 ± 0.03	9.36 ± 0.16	0.18 ± 0.01	0.14 ± 0.01
1.5		1.16 ± 0.03	9.25 ± 0.18	0.17 ± 0.01	0.16 ± 0.01
3.0		1.18 ± 0.02	9.21 ± 0.17	0.15 ± 0.01	0.17 ± 0.01
Se (mg/kg)					
0.0		1.15 ± 0.03	9.39 ± 0.22	0.17 ± 0.01	0.17 ± 0.01
0.2		1.18 ± 0.02	9.25 ± 0.13	0.17 ± 0.01	0.15 ± 0.01
0.4		1.18 ± 0.04	9.17 ± 0.15	0.16 ± 0.01	0.15 ± 0.01
P-values					
WPC		0.846	0.751	0.263	0.085
Se		0.610	0.580	0.677	0.189
WPC × Se		0.072	0.002	0.645	0.040

[1] 45 roosters (five roosters per each group) fed diet containing 3 WPC levels (0.0, 1.5 and 3.0% of diet) and 3 levels of selenium supplement (0.0, 0.2 and 0.4 mg Se/kg of diet) for 8 weeks (62-70 weeks of age).
[a-b] Within each column, values with different superscript(s) differ significantly ($P < 0.05$).

Table 5. The effect of different levels of whey protein concentrate (WPC) and selenium (Se) on liver antioxidant activity and lipid peroxidation[1] (LSmean ± SE)

WPC (%)	Se (mg/Kg)	Glutathione peroxidase (U/mg protein)	Superoxide dismutase (U/mg protein)	Total antioxidants capacity (mmol/L)	Malondialdehyde (nmol/mg protein)
0.0	0.0	0.99 ± 0.07	13.84 ± 1.10	0.59 ± 0.07	0.021 ± 0.002
0.0	0.2	0.99 ± 0.03	13.31 ± 0.56	0.54 ± 0.04	0.024 ± 0.003
0.0	0.4	1.09 ± 0.08	13.82 ± 0.78	0.39 ± 0.03	0.023 ± 0.003
1.5	0.0	1.01 ± 0.03	13.03 ± 0.41	0.54 ± 0.06	0.028 ± 0.004
1.5	0.2	1.07 ± 0.04	14.10 ± 0.39	0.45 ± 0.03	0.024 ± 0.003
1.5	0.4	1.04 ± 0.04	13.38 ± 0.11	0.62 ± 0.05	0.024 ± 0.002
3.0	0.0	1.01 ± 0.03	14.01 ± 0.97	0.47 ± 0.10	0.023 ± 0.003
3.0	0.2	1.10 ± 0.06	12.35 ± 0.36	0.58 ± 0.09	0.019 ± 0.002
3.0	0.4	1.07 ± 0.04	13.41 ± 0.78	0.45 ± 0.09	0.023 ± 0.002
Main effects					
WPC (%)					
0.0		1.03 ± 0.04	13.64 ± 0.46	0.52 ± 0.04	0.022 ± 0.001
1.5		1.04 ± 0.02	13.50 ± 0.21	0.54 ± 0.03	0.026 ± 0.002
3.0		1.06 ± 0.03	13.26 ± 0.44	0.50 ± 0.05	0.022 ± 0.001
Se (mg/kg)					
0.0		1.01 + 0.03	13.63 + 0.48	0.53 ± 0.04	0.024 ± 0.002
0.2		1.06 + 0.03	13.25 + 0.31	0.52 ± 0.03	0.022 ± 0.002
0.4		1.07 + 0.03	13.51 + 0.33	0.50 ± 0.04	0.023 ± 0.001
P-values					
WPC		0.699	0.780	0.817	0.194
Se		0.239	0.779	0.798	0.616
WPC × Se		0.680	0.374	0.124	0.536

[1] 45 roosters (five roosters per each group) fed diet containing 3 levels of WPC (0.0, 1.5 and 3.0% of diet) and selenium supplement (0.0, 0.2 and 0.4 mg Se/kg of diet) for 8 weeks (62-70 weeks of age).

Table 3 exhibits the data for antioxidant enzymes activity and lipid peroxidation in seminal plasma. Glutathione peroxidase and SOD activities were significantly influenced by experimental diets. Selenium at the level of 0.4 mg, and level of 3.0% WPC were associated with significantly increased GPx and SOD activities as compared to other levels ($P < 0.05$). Also, the effect of time and the interaction effect of WPC and Se on GPx and SOD activities were significant ($P < 0.05$). The highest level of TAC was observed at the level of 0.2 mg Se ($P < 0.05$). Also, 3.0% WPC resulted in significantly increased TAC compared to the WPC-free diet ($P < 0.05$). On the other hand, MDA in selenium supplementation-free diets was significantly higher than other levels ($P < 0.05$). Different levels of WPC had no effect on MDA levels.

The data for antioxidant enzymes activity and lipid peroxidation in the testis and liver tissue of broiler breeder roosters are shown in Table 4 and Table 5, respectively. Glutathione peroxidase and SOD activities, TAC, and MDA levels in the testis and liver were not affected by the main effects of WPC and Se levels. ($P > 0.05$). However, SOD activity and MDA concentration in testis tissue was affected by the interaction effect of WPC and Se ($P < 0.05$).

Discussion

Spermatozoa is one of the cells with the highest selenium concentration in the body. Most of the selenium in the testis is concentrated in specific selenoproteins. For instance, sperm mitochondria-associated cysteine-rich protein (SMCP) is a key structural element of the mitochondria in the spermatozoa tail midpiece (Ursini *et al*., 1999). About 50% of SMCP is composed of phospholipid hydroperoxide glutathione peroxidase (GPx), which in turn contains high selenium concentration. The GPx also acts as a powerful antioxidant during spermatogenesis (Ursini *et al*., 1999; Allmang and Krol, 2006). Selenium is a part of the GPx and other selenoproteins contributing to the elimination of hydrogen peroxide and inhibition of lipid peroxides produced during the oxidative process in cells (Ahsan *et al*., 2014). The important role played by GPx during sperm maturation could partially explain the reasons for decreased sperm quality in selenium-deficient animals that are similar to the results observed in this investigation. The results of the present study showed that diets with no selenium supplementation had significantly lower total and progressive sperm motility, sperm concentration, membrane integrity, sperm viability, but higher sperm abnormalities than other treated groups. The results of this study were in agreement with the study of Surai *et al*. (1998) who reported that dietary Se deficiency decreases the numbers of normal spermatozoa per ejaculate and motility in birds. Moreover, it was reported that the absence or deficiency of Se can lead to a decreased concentration of Se in spermatogenic cells. Thus, morphological defects of spermatozoa can occure during spermatogenesis (Kehr *et al*., 2009). According to the study of Edens and Sefton (2009), broiler breeder roosters with no Se source in their diet had a higher abnormal spermatozoa percentage, compared to roosters fed with 0.2 mg/kg sodium selenite or organic selenium. Gallo *et al*. (2003) found that dietary inclusion of Se at 2.5 mg/kg live weight, improved the spermatozoa viability, motility, and concentration. It was also indicated that dietary Se supplementation was beneficial for development of seminiferous tubules and promotion of Sertoli cell viability, which in turn enhances the sperm production (Khalid *et al*., 2016). Furthermore, according to Khalil-Khalili *et al*. (2021), cellular antioxidant capacity and mitochondrial function are improved by Se supplement, which has a key role in sperm viability and motility. Totally, these studies indicate that Se supplementation is vital for development of normal sperm and for enhancing the characteristics of semen quality.

To our knowledge, the effect of WPC on semen quality and oxidative variables, including antioxidant enzyme (GPx, SOD) activities, TAC and MDA in semen has not been assessed previously. However, the effect of WPC on antioxidant enzymes activity and lipid peroxidation in the liver and testis has been investigated. In the present study, Se and WPC supplements increased GPx, SOD and TAC in seminal plasma. Also, Se supplementation alone could reduce lipid peroxidation in seminal plasma. This accords with Ebeid's (2009) postulated that dietary supplementation of Se decreases lipid peroxidation in the seminal plasma mainly through antioxidative properties. It also improves sperm count and motility and reduces dead sperm percentage in breeder males. GPx activity and total antioxidant capacity in the seminal plasma are other traits affected by diets comprising selenium supplements. In addition, Surai *et al*. (1998) revealed an association between the incremented activity of GPx in seminal plasma obtained from birds fed with the Se-supplemented diet and the higher protection against lipid peroxidation in semen. They reported that lipid peroxidation in the seminal plasma was reduced by including Se (0.3 mg/kg) in the male breeders' diet. The positive effects of Se observed in this study can be explained by the vital role of Se in incrementing the antioxidant capacity that protects the seminal plasma against ROS and consequently lowers the MDA content.

Our study showed that GPx and SOD activities along with TAC and MDA levels in the liver and testis were not affected by different levels of WPC and Se. In contrast with our findings, Ashour *et al*.

(2019) reported that with increasing dietary WPC levels, a gradual reduction in MDA values and elevation in GPx, and SOD in the liver of broilers were recorded. Moreover, In contrast with our results Surai *et al*. (1998) reported that the inclusion of the Se into the diet of cockerels (28-wk-old) had a significant stimulating effect on GPx activity in the testis and liver. Afkhami *et al*. (2020) demonstrated that dietary inclusion of WPC (2%) increased levels of liver antioxidant enzymes (GPx and SOD) activities, butdecreased MDA in broiler chickens. The discrepancy observed between our results and previous studies may be due to the use of aged roosters (60-wk-old) in the present study. Because it has been reported that antioxidant enzyme activity, mainly of GPx, is reduced significantly with age (Jervis and Robaire, 2002). It was also indicated that the GPx activity is decreased by half in aged rats compared to their younger generation (Weir and Robaire, 2007).

Based on the data presented above and taking into account the role of antioxidant enzymes, it is possible to suggest that dietary supplementation of WPC and Se could improve semen quality and reduce lipid peroxidation in the seminal plasma of roosters.

Conclusion

In the present study, dietary supplementation of WPC (1.5%) and Se (0.2 mg/kg of diet) improved sperm kinematic parameters, plasma membrane integrity, enzymatic antioxidant activity and lipid peroxidation in male broiler breeders. However, further studies are required to collect fertility data and also to confirm the obtained results.

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