

Natural Astaxanthin From *Haematococcus Pluvialis* Enhanced Antioxidant Capacity And Improved Semen Quality In Aging Layer Breeder Roosters Through The Mitogen-Activated Protein Kinase/Nuclear Factor-Erythroid 2-Related Factor 2 (MAPK/Nrf2) Pathway

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Abstract

Background: Natural astaxanthin (ASTA) has strong antioxidant properties and has been widely used as a health product to improve human health. However, the effects of ASTA on the reproductive performance of aging roosters have been poorly studied. We aimed to investigate the effects of dietary ASTA on semen quality and antioxidant capacity in aging roosters and to explore the potential mechanism of semen quality change via anti-oxidation defense system.

Methods: In the present study, 96 53-week-old Jinghong No. 1 layer breeder roosters were fed a corn-soybean meal basal diet containing ASTA at 0, 25, 50, or 100 mg/kg for 6 week.

Results: Semen quality in the ASTA groups remarkably improved as compared to those in the control group and antioxidant activities, the abilities to scavenge hydroxyl radicals and superoxide anions increased gradually with ASTA addition ($P < 0.05$). In addition, the mRNA levels of antioxidant enzymes, the mRNA and protein levels of the mitogen-activated protein kinase (MAPK), nuclear factor-erythroid 2-related factor 2 (Nrf2) were markedly increased in the 50-100 mg/kg ASTA group ($P < 0.05$).

Conclusions: Collectively, these results demonstrate that dietary ASTA may improve semen quality by increasing antioxidant enzyme activities, and the ability to scavenge hydroxyl radicals, which may be related to up-regulation of the MAPK/Nrf2 pathway.

Introduction

In poultry production, assisted reproductive technology such as artificial insemination is widely used. A single male is responsible for fertilizing dozens of female birds[1]. Therefore, the reproductive performance of roosters is a key factor in production. However, the sperm quality of roosters decreases gradually at approximately 50–55 weeks[2]. Several important reproductive traits change in aging roosters, including decreases in the sperm concentration, motility, testosterone level, and antioxidant capacity[3; 4]. Male fertility is one of the most important factors in determining hatchability. Therefore, it is necessary to find a way to alleviate the influence of age on the reproductive characteristics of breeding roosters. Recently, studies have shown that dietary supplementation with natural anti-oxidative compounds such as dietary linseed oil[5], lycopene[6] and curcumin[7] can alleviate the negative effects of age on roosters.

Astaxanthin (3,3-dihydroxy- β , β -carotene-4,4-dione) is a xanthophyll carotenoid, that is mainly divided into two forms: natural astaxanthin (ASTA) and synthetic astaxanthin. ASTA can be extracted from algae[8], yeast[9], fish, krill, and some microorganisms[10]. The microalgae *Haematococcus pluvialis* (a green microalga) is one of the major sources of ASTA, and can be extract a large amount of ASTA under stress conditions[12; 11]. Although synthetic astaxanthin has the same chemical formula as ASTA, the ability to scavenge singlet oxygen and free radical of ASTA is over 50 times and 20 times that of synthetic astaxanthin, respectively [13]. Meanwhile, the European Commission considers ASTA as a food dye[14]. Currently, ASTA has received much attention for its various beneficial characteristics, including its anti-oxidant[15], anti-inflammatory[16] and anti-apoptotic properties[17]. However, there is little information on whether ASTA can improve the male reproductive performance.

The mitogen-activated protein kinase (MAPK) pathway, a family of serine/threonine protein kinases, is widely conserved in eukaryotes and is involved in antioxidant enzyme expression by modulating the Nrf2/ARE axis[18; 19]. Nuclear factor-erythroid 2-related factor 2 (Nrf2) is an important regulator of antioxidant responsive element (ARE)-activated gene expression. Nrf2 can repair and degrade of damaged macromolecules caused by oxidative stress by upregulating the expression of antioxidant enzyme genes[20]. Studies have shown that the MAPK/Nrf2 pathway improves the antioxidant capacity of eggs by upregulating the mRNA expression of P38MAPK, Nrf2, and HO-1[21]. Moreover, the Nrf2/ARE axis alleviates meat lipid peroxidation by regulating the expression of antioxidant enzymes and phase II enzymes in order to achieve long-term meat storage[22]. However, the potential effect of ASTA on sperm quality, antioxidant capacity, and the MAPK/Nrf2 antioxidant pathway in aging layer roosters was not clear.

Therefore, in the current study, we hypothesized that ASTA can improve semen quality by enhancing antioxidant capacity and activating the MAPK/Nrf2 pathway in aging roosters.

Materials And Methods

Animal care and use

All experimental protocols were approved by the Animal Care and Use Committee of the Beijing University of Agriculture.

Experimental design

In the present study, a total of ninety-six 53-week old Jinghong No.1layer breeder roosters with a similar weight and genetic background were supplied by Beijing Huadu Group Co., Ltd. (Beijing, China), and the birds were randomly distributed in four treatments, following a completely randomized design. Each treatment had six replicates, and there were four birds per replicate with one bird per cage (70 cm × 60 cm × 75 cm). All birds were fed a basal diet for 1week and then assigned to a corn-soybean meal-based diet containing 0, 25, 50, and 100 mg/kg ASTA for 6 weeks. *Haematococcus pluvialis* was purchased from Jingzhou Natural Astaxanthin Inc. (Jingzhou, China), and the ASTA content was 1.54%. The composition and nutrient levels of the corn–soybean meal-based diet are shown in Table 1. During the experimental period, the birds had 16-h light cycles and were allowed ad libitum access to water and the treatment diets.

Table 1
Composition and nutrient content of basal diets (air dried basis, %)

Ingredients	Content (%)	Nutrition level	
Corn	69.93	Metabolizable energy /(MJ·kg ⁻¹)	12.02
Soybean meal	18.60	Crude protein/%	14.00
Wheat bran	3.80	Methionine/%	0.23
Soybean oil	1.40	Lysine/%	0.64
Limestone	2.60	Calcium/%	1.37
Dicalcium phosphate	1.80	Total phosphorus/%	0.63
Salt	0.25	Available phosphorus/%	0.44
Choline chloride	0.20		
Premix ¹	1.42		
Total	100.00		
¹ The premix provided the following per kilogram of the diet: Cu 10 mg, Fe 80 mg, Mn 100 mg, Zn 80 mg, VA 20,000 IU, VD 3,000 IU, VE 30 IU, VK 2 mg, VB1 2 mg, VB2 10 mg, VB6 6 mg, VB12 0.012 mg, folic acid 1.2 mg, D-pantothenic acid 12 mg, nicotinic acid 40 mg, biotin 0.2 mg, and Se 0.3 mg.			

Semen collection and analysis

Birds were trained for semen collection every 2 weeks. Semen samples were randomly collected from each bird in each replicate once every two weeks using the abdominal massage method. Next, semen volume was measured with a graduated collecting tube. Semen samples were diluted in a pre-warmed (37 °C) saline solution (0.9%, 1:49), after which samples were assessed for seminal characteristics using CASA system (WLJX-9000 Weili Color Sperm Analysis System; Weili New Century Science & Tech Dev., Beijing, China), including semen concentration and sperm motility parameters such as sperm motility (%), average path velocity (VAP, μm/s), straight linear velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), straightness (STR, %), linearity (LIN, %), wobble of the curvilinear trajectory (WOB, %) and beat cross frequency (BCF, Hz).

Sperm viability was evaluated using the eosin-nigrosin stain method. Briefly, a 5 μL sperm sample was mixed with 25 μL of stain on a pre-warmed slide before the test. Then, the stain was spread with another slide to make a sperm suspension smear. An Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan) was used at a final magnification of 400 to analyze the sperm

viability. For each sperm sample, five microscopic fields were observed subjectively, and 200 sperms were counted to evaluate viability. Sperm with unstained heads of spermatozoa were considered viable, while sperm displaying partial or complete purple staining were considered nonviable.

Antioxidant enzymes and oxygen free radicals analysis

After analyzing the semen quality, the semen sample was centrifuged at $4000 \times g$ for 10 min at 4 °C, and then the seminal plasma was separated and stored at -20 °C. At the end of the 6-week feeding trial, one bird from each replicate was randomly selected and sacrificed by cervical dislocation. Immediately after euthanasia, blood samples were collected from the left jugular vein of one bird per replicate. Plasma was separated by centrifugation at $3000 \times g$ for 10 min and stored at -20 °C until analysis. The testis, liver, and kidney were removed immediately and quickly frozen at -80 °C for later analysis. These samples were used to analyze the activities of antioxidant enzymes and the ability to scavenge oxygen free radicals. Briefly, antioxidant capacity included the glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT), and total antioxidant capacity (T-AOC), while oxygen free radical scavenging abilities included hydroxyl radical scavenging ability and superoxide anion scavenging ability. Meanwhile, malondialdehyde (MDA), as the product of lipid metabolism, was analyzed using a spectrophotometric method. These assays were performed using a commercial kit (Nanjing Jiancheng Bio-Engineering Institute, Nanjing, China). The method and principle used to determine the antioxidant indicators with these kits were previously described elsewhere [23].

Quantitative PCR Analysis

The mRNA expression levels of *p38*, extracellular signal-regulated kinase (*ERK*), c-Jun N-terminal kinase 1 (*JNK1*), c-Jun N-terminal kinase 2 (*JNK2*), c-Jun N-terminal kinase 3 (*JNK3*), *Nrf2*, Cu-Zn superoxide dismutase (*SOD1*), Mn superoxide dismutase (*SOD2*), *CAT*, glutathione peroxidase 1 (*GPX1*), and peroxidase 4 (*GPX4*) were determined via real-time quantitative PCR (RT-qPCR) with β -actin (*ACTB*) as an internal control in the testis tissues. Total RNA was isolated from 0.1 g of the testis sample using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Then, 1% agarose gel stained with ethidium bromide was used to confirm the 18S and 28S bands. Finally, the total RNA concentration was measured using a spectrophotometer at 260 nm. Total RNA was reverse transcribed into cDNA using a Thermo Fisher First cDNA Synthesis Kit (#K1621; Thermo Fisher Scientific, Waltham, MA, USA). A Step One Plus Real-time PCR system (Applied Biosystems, Foster City, CA, USA) was used for the quantitative PCR analyses. The primers for the selected genes are listed in Table 2. After initial denaturation at 95 °C for 10 min, 40 cycles of amplification were performed (95 °C for 10 s and 58 °C for 30 s), generating melt curves to verify amplification specificity. The relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method[24], with *ACTB* as the reference gene.

Table 2
Primers used for the real-time quantitative PCR

Genes	Primer sequence (5'-3')	Fragment length (bp)		Annealing temperature (°C)	Accession number
<i>ACTB</i>	Forward: GCCAACAGAGAGAAGATGACAC Reverse: GTAACACCATCACCAGAGTCCA	118	58		NM_205518
<i>SOD1</i>	Forward: TTGTCTGATGGAGATCATGGCTTC Reverse: TGCTTGCCTTCAGGATTAAAGTGAG	98	58		NM_205064
<i>SOD2</i>	Forward: CAGATAGCAGCCTGTGCAAATCA Reverse: GCATGTTCCCATACATCGATTCC	86	58		NM_204211.1
<i>CAT</i>	Forward: ACCAAGTACTGCAAGGCGAAAGT Reverse: ACCCAGATTCTCCAGCAACAGTG	91	58		NM_001031215.2
<i>GPX-1</i>	Forward: TTCGAGAAGTTCCTCGTGGG Reverse: CCTGCAGTTTGATGGTCTCG	79	58		NM_0012778553.2
<i>GPX-4</i>	Forward: TCAACCGTGAGGGCCAAGT Reverse: CTCGGCACGCAGCTCTAC	100	58		NM_001346448.1
<i>Nrf2</i>	Forward: ACATGGACAGTTCTCCTGGG Reverse: CGGCTCCACAGAAGGAAGTA	92	58		NM_205117.1
<i>ERK</i>	Forward: AGCAAGCTTTAGCCCATCCA Reverse: CCTTCGGCAAGTCATCCAAT	108	58		NM_204150.1
<i>JNK1</i>	Forward: GGGTGCATTATGGGCGAAAT Reverse: TTCTGGGCACGGTGTTCTTA	108	58		XM_421650.2
<i>JNK2</i>	Forward: AGCAGCCTCGATGCCTTGAC Reverse: CAAGCAATTCAGGCCCAATG	110	58		AB000807.1
<i>JNK3</i>	Forward: CTGGTGAGTGAGCTGATGGA Reverse: ACAGCAGGTAGGACATTCTG	82	58		NM_001318224.3
<i>p38</i>	Forward: TGTGTTCACCCCTGCCAAGT Reverse: GCCCCCGAAGAATCTGGTAT	149	58		AJ719744.1

Western blotting analysis

The protein expression of Nrf2, p38, p-p38, ERK, p-ERK, JNK, and p-JNK was evaluated by western blotting in the testis tissues, and the antibodies are shown in Table 3. Total proteins were extracted from testis tissues using RIPA Buffer (W1001; SinoGene, Beijing, China) according to the manufacturer's instructions. The protein concentration of the extracts was determined using the Bradford method (W1014; SinoGene). Samples containing 30 µg of protein and protein markers (SM26616; Thermo Fisher Scientific) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 4% stacking gel, 12% separating gel) at 120 V for 2 h. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes using a Bio-Rad mini transfer system (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with Fast Protein-free Block Buffer (#W1028; SinoGene) for 5 min and then incubated with primary antibody (1:1000 dilution) overnight at 4 °C. After rinsing four

times with TBST for 10 min per rinse, HRP-conjugated secondary antibody (SA00001-2, ptg) was applied to the membranes at a dilution of 1:3000 for 1 h. Then, after rinsing three times with Tris-buffered saline with 0.1% Tween ® 20 Detergent (TBST) for 10 min per rinse, immunological signals were detected using a chemiluminescent (ECL) kit (29050; Engreen) and exposed to X-ray films in the dark. Protein bands were quantified by densitometric analysis using Image J analysis software (National Institutes of Health, Bethesda, MD, USA).

Table 3
Antibodies used for the western blot analysis.

Antibodies	Cat NO.	Source	Dilution
p38	9212	Cell Signaling Technology, Danvers, MA, USA	1:1000
p-p38	4511	Cell Signaling Technology, Danvers, MA, USA	1:1000
ERK	4695	Cell Signaling Technology, Danvers, MA, USA	1:1000
p-ERK	9101	Cell Signaling Technology, Danvers, MA, USA	1:1000
JNK	9252	Cell Signaling Technology, Danvers, MA, USA	1:1000
p-JNK	4668	Cell Signaling Technology, Danvers, MA, USA	1:1000
Nrf2	ab31163	Abcam, Cambridge, UK	1:5000
β-Actin	AC028	Abclonal, Woburn, MA, USA	1:3000

Statistical analysis

Statistical analysis was performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA). Data related to the effect of dietary ASTA levels on semen quality, antioxidant capacity, gene and protein expression were analyzed by one-way analysis of variance with orthogonal linear and quadratic contrasts. Duncan's multiple comparison test was used to examine statistical differences among the treatments. Statistical significance was defined as $P < 0.05$.

Results And Discussion

Semen quality

Semen quality is considered to be a crucial factor in roosters' fertility because the fertility rate is positively correlated with sperm motility, sperm concentration, and sperm motility parameters in roosters[25]. Few reports have described the effects of ASTA on semen quality in aging roosters. We evaluated the effects of dietary ASTA on semen quality in aging roosters (Table 4). Briefly, there were no differences in semen quality between the control group and the ASTA groups at week 0. The semen volume was significantly higher in the 25 mg/kg ASTA group than that in the other groups at week 6 ($P < 0.05$). Meanwhile, with an increase in dietary ASTA, sperm viability and sperm concentration significantly increased compared to those in the control group ($P < 0.05$). In particular, the sperm concentration was linearly and quadratically affected ($P < 0.05$) by the dietary ASTA levels. In addition, the sperm motility parameters of aging layer roosters in the 50 mg/kg ASTA group were significantly linearly increased relative to those in the control group ($P < 0.05$). Moreover, sperm motility increased from 64.395–76.234% in the 0–100 mg/kg ASTA-treated group ($P < 0.05$). It has been previously demonstrated that sperm viability, total motility, and sperm kinematic parameters increase after ASTA treatment in mice[26]. A recent study also showed similar results: ASTA can improve post-thawed rooster sperm motility and kinetic parameters, including total motility, progressive motility, VAP, VSL, and LIN. However, there was no significant difference in LIN, STR, ALH, and BCF in the ASTA treatment groups compared to those in the control[27]. Interestingly, the opposite result was observed in the current study that the motility parameters including STR, LIN, WOB, VSL, VAP, BCF, and ALH in the ASTA groups were significantly higher than those in the control group ($P < 0.05$). The discrepant results may be partly related to the level of ASTA addition, the duration of supplementation, and the test subjects. Taken together, these findings indicated that dietary ASTA supplementation significantly increased the semen quality during the test period (0–6 weeks). However, the possible mechanisms by which dietary ASTA affects semen quality are not well understood, but may be related to antioxidant defense system.

Table 4
Effect of dietary natural astaxanthin (ASTA) supplementation on the semen quality of aging layer breeder roosters

Items	Time (week)	ASTA levels / (mg/kg)				SEM	P-value		
		0	25	50	100		ANOVA	Linear	Quadratic
Semen volume (mL)	0	0.245	0.258	0.255	0.242	0.005	0.606	0.769	0.199
	2	0.252 ^b	0.309 ^a	0.274 ^b	0.274 ^b	0.006	< 0.01	0.429	< 0.01
	4	0.242 ^b	0.305 ^a	0.289 ^a	0.286 ^a	0.006	< 0.01	< 0.01	< 0.01
	6	0.252 ^b	0.336 ^a	0.317 ^a	0.31 ^a	0.054	< 0.01	< 0.01	< 0.01
Sperm viability (%)	0	83.83	81.33	82.17	82.83	0.42	0.185	0.549	0.06
	2	85.33 ^b	89.17 ^a	90.83 ^a	90.83 ^a	0.65	< 0.01	< 0.01	0.063
	4	83.17 ^c	88.67 ^b	90.17 ^{ab}	91.50 ^a	0.75	< 0.01	< 0.01	0.011
	6	85.67 ^c	91.33 ^{ab}	91.00 ^b	94.67 ^a	0.87	< 0.01	< 0.01	0.40
Sperm concentration(10 ⁸ /mL)	0	11.357	11.278	11.303	11.366	3.148	0.742	0.862	0.291
	2	11.369 ^b	11.489 ^b	11.844 ^a	11.956 ^a	5.427	< 0.01	< 0.01	0.931
	4	11.374 ^c	12.036 ^b	12.154 ^b	12.421 ^a	8.489	< 0.01	< 0.01	< 0.01
	6	11.33 ^c	12.569 ^b	12.623 ^b	12.965 ^a	13.523	< 0.01	< 0.01	< 0.01
STR (%)	0	79.764	79.725	79.51	79.323	0.783	0.997	0.839	0.965
	2	78.082 ^c	84.222 ^b	87.144 ^a	84.772 ^{ab}	0.815	< 0.01	< 0.01	< 0.01
	4	79.236 ^c	84.269 ^b	87.377 ^a	86.635 ^a	0.747	< 0.01	< 0.01	< 0.01
	6	76.534 ^b	85.283 ^a	86.743 ^a	87.413 ^a	1.013	< 0.01	< 0.01	< 0.01
LIN (%)	0	44.781	44.568	45.51	43.034	0.429	0.224	0.259	0.186
	2	45.156 ^c	53.476 ^b	55.586 ^a	55.955 ^a	0.99	< 0.01	< 0.01	< 0.01
	4	48.07 ^c	54.102 ^b	57.377 ^a	57.468 ^a	0.857	< 0.01	< 0.01	< 0.01
	6	45.467 ^c	61.142 ^a	61.078 ^a	58.087 ^b	1.43	< 0.01	< 0.01	< 0.01
WOB (%)	0	52.731	53.159	53.065	52.091	0.408	0.811	0.604	0.422
	2	53.903 ^c	58.392 ^b	59.617 ^b	63.089 ^a	0.787	< 0.01	< 0.01	0.542
	4	54.267 ^c	61.104 ^{ab}	59.132 ^b	63.069 ^a	0.774	< 0.01	< 0.01	0.08
	6	52.961 ^c	69.569 ^a	71.067 ^a	64.15 ^b	1.62	< 0.01	< 0.01	< 0.01
VCL (μm/s)	0	63.226	61.61	62.294	62.57	0.396	0.572	0.726	0.256
	2	62.403 ^c	67.73 ^b	76.292 ^a	69.393 ^b	1.099	< 0.01	< 0.01	< 0.01
	4	60.631 ^d	70.735 ^c	80.973 ^b	85.911 ^a	2.142	< 0.01	< 0.01	0.096

^{a-d} Means within a row with no common superscripts differ significantly ($P < 0.05$). Data represent the mean of six replicates. Abbreviations: SEM, standard error of the mean; ANOVA, analysis of variance; VAP, average path velocity; VSL, straight linear velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; STR, straightness; LIN, linearity; WOB, wobble of the curvilinear trajectory; BCF, beat cross frequency.

	6	59.894 ^c	73.602 ^b	80.763 ^a	83.072 ^a	2.009	< 0.01	< 0.01	< 0.01
VSL (µm/s)	0	29.595	29.752	29.381	28.56	0.447	0.81	0.416	0.607
	2	31.673 ^c	32.719 ^{bc}	36.371 ^b	46.35 ^a	1.354	< 0.01	< 0.01	< 0.01
	4	30.82 ^c	38.916 ^b	41.511 ^b	45.976 ^a	1.268	< 0.01	< 0.01	0.132
	6	30.024 ^b	40.53 ^a	37.129 ^a	41.164 ^a	1.198	< 0.01	< 0.01	0.072
VAP (µm/s)	0	32.264	31.996	31.056	32.379	0.406	0.834	0.939	0.458
	2	31.192 ^d	35.268 ^c	41.78 ^b	47.127 ^a	1.332	< 0.01	< 0.01	0.468
	4	32.999 ^c	44.309 ^b	45.593 ^b	53.379 ^a	1.629	< 0.01	< 0.01	0.148
	6	32.093 ^c	46.592 ^a	47.366 ^a	41.523 ^b	1.439	< 0.01	< 0.01	< 0.01
BCF (Hz)	0	3.568	3.53	3.672	3.545	0.032	0.423	0.802	0.498
	2	3.534 ^c	3.81 ^b	3.97 ^a	4.047 ^a	0.045	< 0.01	< 0.01	0.024
	4	3.528 ^c	4.068 ^b	4.60 ^a	4.141 ^b	0.084	< 0.01	< 0.01	< 0.01
	6	3.59 ^c	3.889 ^b	4.44 ^a	4.387 ^a	0.079	< 0.01	< 0.01	< 0.01
Sperm motility (%)	0	63.072	61.520	63.000	61.948	0.502	0.649	0.687	0.811
	2	62.554 ^b	65.200 ^{ab}	67.951 ^a	66.508 ^a	0.615	< 0.01	< 0.01	0.049
	4	62.000 ^c	68.833 ^b	69.779 ^{ab}	71.667 ^a	0.851	< 0.01	< 0.01	< 0.01
	6	64.395 ^b	65.768 ^b	73.414 ^a	76.234 ^a	1.229	< 0.01	< 0.01	0.611
ALH (µm)	0	2.195	2.282	2.180	2.203	0.145	0.645	0.776	0.602
	2	2.122 ^c	2.307 ^{bc}	2.462 ^b	2.962 ^a	0.076	< 0.01	< 0.01	0.082
	4	2.346 ^b	3.099 ^a	3.045 ^a	3.295 ^a	0.087	< 0.01	< 0.01	0.015
	6	2.105 ^c	2.998 ^b	3.018 ^b	3.361 ^a	0.107	< 0.01	0.011	< 0.01

^{a-d} Means within a row with no common superscripts differ significantly ($P < 0.05$). Data represent the mean of six replicates. Abbreviations: SEM, standard error of the mean; ANOVA, analysis of variance; VAP, average path velocity; VSL, straight linear velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; STR, straightness; LIN, linearity; WOB, wobble of the curvilinear trajectory; BCF, beat cross frequency.

Antioxidant enzyme activity and free radical scavenging ability

In aging roosters, a decreased in the antioxidant defense system, makes rooster spermatozoa vulnerable to lipid peroxidation[28]. Other studies have reported that senescence occurs due to the damage caused by free radicals and reactive oxygen species (ROS) to DNA, lipids, and proteins[29; 30]. Sophisticated enzymatic (SOD, CAT and GSH-Px) and non-enzymatic (vitamins A, C, E, and carotenoids) antioxidants constitute an antioxidant defense system that can regulate overall ROS levels to maintain physiological homeostasis[31]. ASTA, as a natural carotenoid antioxidant, can be transferred to the right place in the tissues and exert antioxidant effects at an appropriate concentration[32]. Previous studies have demonstrated that ASTA has a higher antioxidant activity relative to α -carotene, lycopene, lutein, and β -carotene[33], and the capacity of scavenging singlet oxygen is about 550 times more than that of vitamin E[34]. Thus, in this study, the effects of dietary ASTA addition on antioxidant enzyme activity and free radical scavenging ability in the plasma, seminal plasma, testes, liver, and kidney of aging layer breeder roosters are shown in Figures 1 to 5. Briefly, the 25 mg/kg ASTA level linearly and quadratically increased the GSH-Px activity in the plasma, testis, and liver of aging layer roosters compared with that in the control group ($P < 0.05$). The activities of SOD and CAT also increased gradually with increasing dietary ASTA supplementation from 50 to 100 mg/kg ($P < 0.05$). Furthermore, MDA

concentrations decreased linearly with increasing dietary ASTA levels ($P < 0.05$) in birds. With an increase in dietary ASTA, the levels of T-AOC increased linearly ($P < 0.05$). This observation is consistent with a previous report that in general, the activities of SOD, CAT, and GSH-Px in the brain and liver of rats increased dramatically at treatment with ASTA, respectively[36; 35]. In addition, we found that dietary ASTA supplementation linearly and quadratically increased the abilities of scavenging hydroxyl radicals and superoxide anions compared with those in the control group, which was consistent with a previous study[37]. In particular, the abilities of scavenging hydroxyl radical and superoxide anion of aging roosters were significantly increased in the 50 mg/kg ASTA group ($P < 0.05$).

Relationship between semen quality and antioxidant enzyme activity

When the age of male poultry is over 45 weeks, semen volume, sperm concentration, viability, and forward motility decrease, whereas lipid peroxidation in seminal plasma increases[38]. Therefore, in the present study, we aimed to improve the quality of semen by improving the antioxidant capacity of aging roosters using dietary ASTA. Moreover, it has been demonstrated that antioxidant capacity plays an important role in maintaining sperm viability because it can protect against lipid peroxidation in semen[39]. MDA is an important indicator of lipid peroxidation. A previous study showed that sperm motility was negatively correlated with MDA content in spermatozoa[40]. In the current study, the MDA content in the semen plasma, plasma, and tissues showed a dose-dependent decrease with changes in dietary ASTA. Similar findings were obtained in rats in which ASTA group showed a significant increase in SOD and CAT activities, as well as a significant decrease in MDA levels and repaired the oxidative stress-induced fertility disorder[41]. Therefore, the improvement of semen quality in aging roosters may be related to two factors. First, antioxidant enzyme activity remarkably improved, and MDA concentration decreased linearly with increasing dietary ASTA concentration, which plays an important role in the prevention of oxidative damage in the sperm plasma membrane. Second, dietary ASTA may improve the ability to neutralize singlet oxygen and scavenge radicals to inhibit lipid peroxidation, protect sperm from free radicals, and improve semen quality.

Gene expression of *SOD1*, *SOD2*, *CAT*, *GPX1*, and *GPX4*

The activity of an antioxidant enzyme is closely related to its gene expression[42]. The mRNA levels of *SOD1*, *SOD2*, *CAT*, *GPX1*, and *GPX4* were determined to evaluate the effects of ASTA on the gene expression of antioxidant enzymes in aging rooster testis tissues (Figure 6). In the present study, the *SOD1* and *SOD2* mRNA levels in the 50 mg/kg ASTA groups were higher than those in the other groups ($P < 0.05$). SOD1 is mainly soluble in the cytoplasm and SOD2 is mitochondrial, and ASTA has both lipophilic and hydrophilic properties, which can directly produce effects in or outside the cell membrane[43]. This may be the reason why the gene expression of *SOD1* and *SOD2* was improved by adding dietary ASTA. Additionally, in the dietary ASTA supplementation group, the *GPX1* and *GPX4* mRNA levels were significantly increased relative to those in the control group ($P < 0.05$). However, there were no differences in the *GPX1* mRNA levels among the 50 and 100 mg/kg ASTA-treated groups. Furthermore, an increase in dietary ASTA supplementation from 0 to 100 mg/kg linearly increased the mRNA expression of *CAT* ($P < 0.05$). Similarly, in a previous study, dietary ASTA increased the oxidative damage repair potential of mice and upregulated the mRNA levels of *GPX1*, *SOD1*, *SOD2*, and *CAT* in the liver and kidney[44]. Therefore, it would be interesting to further study the mechanism of how dietary ASTA affects semen quality in aging roosters by improving the antioxidant defense system.

Gene and protein expression in the MAPK/Nrf2 pathway

Nrf2 plays an important role in the antioxidant response, and it has been reported that the MAPK kinase pathway regulates the Nrf2 action. When the nuclear transcription factor Nrf2 is phosphorylated and activated by MAPKs (such as ERK and p38), it can be translocated to the nucleus, leading to the upregulation of antioxidant enzyme expression[22]. Therefore, in order to clarify whether ASTA could activate this pathway, the gene and protein expression in the MAPK/Nrf2 pathway in testis tissues were measured in the four groups, and the results are presented in Figures 7 and 8. In this study, significant differences in the mRNA levels of *Nrf2*, *ERK*, *P38*, and *JNK* were observed among all of the ASTA-added groups in the testis (Figure 7). Briefly, when the concentration of dietary ASTA reached 50 mg/kg, the mRNA levels of *Nrf2*, *ERK*, *P38*, and *JNK2* were significantly higher than those in the control group ($P < 0.05$) and reached the maximum. A significant increase in *JNK1* mRNA expression was observed in the 100 mg/kg ASTA group ($P < 0.05$). Moreover, an increase in dietary ASTA from 0 to 100 mg/kg linearly enhanced the mRNA expression of *JNK3* ($P < 0.05$). This finding indicated that dietary ASTA increased the gene expression of *Nrf2* and upregulated

the expression of upstream signals (MAPKs). Western blot analysis revealed that dietary ASTA effectively elevated ($P < 0.05$) the ratio of the phosphorylated MAPKs (p38, ERK, and JNK) to total MAPKs (p38, ERK, and JNK) compared to that in the control (Figure 8). Briefly, the results showed that ASTA treatment remarkably upregulated the p-p38, p-ERK, and p-JNK expression in the testis ($P < 0.05$). In particular, the p-p38, p-JNK, and Nrf2 protein levels in the 25 mg/kg ASTA group were higher than those in the other groups. In addition, when the concentration of dietary ASTA reached 50 mg/kg, p-ERK protein levels were significantly higher than those in the control group ($P < 0.05$) and reached the maximum. However, Niu et al., 2018 [45] observed an opposite result in that dietary ASTA supplementation did not affect the p-38 and p-JNK levels but significantly increased the p-ERK levels. Differences in test subjects, and test conditions may explain these discrepant results.

Relationships among the MAPK/Nrf2 pathway and antioxidant enzymes and semen quality

Aging is a complex phenomenon that is associated with an irreversible and progressive decline in body function due to biochemical and morphological changes[46]. In the process of aging, there are obvious characteristics such as increase in oxidative stress, a decline in cell function, and defects in tissues and organs[47]. In aging male animals, morphological changes in the testis include a reduction in the volume and quantity of germ cells, which leads to a decrease in sperm quality and fertilization[48]. Therefore, in this study, we explored whether dietary ASTA can improve the semen quality of aging roosters by activating the MAPK/Nrf2 pathway to enhance the service life of aging roosters. The MAPK signaling pathway is involved in many cellular functions, such as inflammation, cell proliferation, cell differentiation, and cell death[18]. Meanwhile, the MAPK signaling pathway can regulate the expression of antioxidant enzymes in various cell types and adapt to various extracellular stresses through the Nrf2/ARE axis[49]. Nrf2 is a major factor that regulates antioxidant responses, and dissociates from Keap1 upon the oxidative response and translocates to the nucleus, wherein it is phosphorylated at serine 40 by the MAPK pathway, leading to the upregulation of the gene expression of antioxidant enzymes[50]. There is a high proportion of polyunsaturated fatty acids in the plasma membrane of bird sperm, and with an increase in age, the antioxidant capacity of sperm decreases. Once the plasma membrane of bird sperm is exposed to ROS, it is prone to lipid peroxidation [51]. Thus, the decrease in total antioxidant capacity of semen is considered to be one of the reasons for the decline of fertilization ability in aging roosters[52]. In the current study, our results revealed that dietary ASTA elevated the MAPK phosphorylation (p38, ERK, JNK), and the mRNA and protein expression of Nrf2 were remarkably enhanced by adding 50 mg/kg ASTA in the diets, which was in agreement with a previous study[53]. In addition, the *SOD1*, *SOD2*, *CAT*, *GPX1*, and *GPX4* mRNA levels were higher in the dietary ASTA group than those in the control group, which led to the enhancement of antioxidant capacity and semen quality in aging roosters. These results indicate that dietary ASTA can activate the MAPK/Nrf2 pathway, upregulate Nrf2 transcription and translation, and promote the expression of downstream antioxidant enzyme genes, enhancing the antioxidant capacity and improving the semen quality in aging roosters.

Conclusions

In summary, the results of this study confirmed our hypothesis that dietary ASTA supplementation improves the semen quality of aging roosters, as reflected by the upregulation of the antioxidant system (Fig. 9). Therefore, these findings suggest that dietary ASTA could attenuate age-related sub-fertility in aging layer breeder roosters. However, the possible mechanisms by which ASTA ameliorates sperm quality are not well understood. Therefore, it is necessary to evaluate the effect of dietary ASTA on semen quality by the MAPK/Nrf2 signaling pathway in vitro.

Abbreviations

ACTB, β -actin; ALH, amplitude of lateral head displacement; ARE, antioxidant responsive element; ASTA, natural astaxanthin; BCF, beat cross frequency; CAT, catalase; GPX1, glutathione peroxidase 1; GPX4, peroxidase 4; GSH-Px, glutathione peroxidase; ERK, extracellular signal-regulated kinase; JNK1, c-Jun N-terminal kinase 1; JNK2, c-Jun N-terminal kinase 2; JNK3, c-Jun N-terminal kinase 3; LIN, linearity; MAPK, mitogen-activated protein kinase; MDA, malondialdehyde; Nrf2, Nuclear factor-erythroid 2-related factor 2; SOD, superoxide dismutase; SOD1, Cu-Zn superoxide dismutase; SOD2, Mn superoxide dismutase; STR, straightness; T-AOC, total antioxidant capacity; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight linear velocity; WOB, wobble of the curvilinear trajectory.

Declarations

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Availability of data and materials

The data for the current study are available from the corresponding author upon reasonable request.

Authors' contributions

SG, XLQ, NH, and FL conceived and designed the experiments. SG performed animal experiments, analyzed the data and wrote the manuscript. XLQ, YG, and HMN assisted with data analysis and paper writing. YC and LW supervised and provided continuous guidance for the animal experiment. XHS, XGW, KX, and LFX supervised and provided continuous guidance for the Molecular experiment. All authors read and approved the final manuscript.

Ethics approval

All experimental protocols were approved by the Animal Care and Use Committee of the Beijing University of Agriculture.

Consent for publication

Not applicable.

Competing interests

The authors have no conflicts of interest to declare.

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Figures

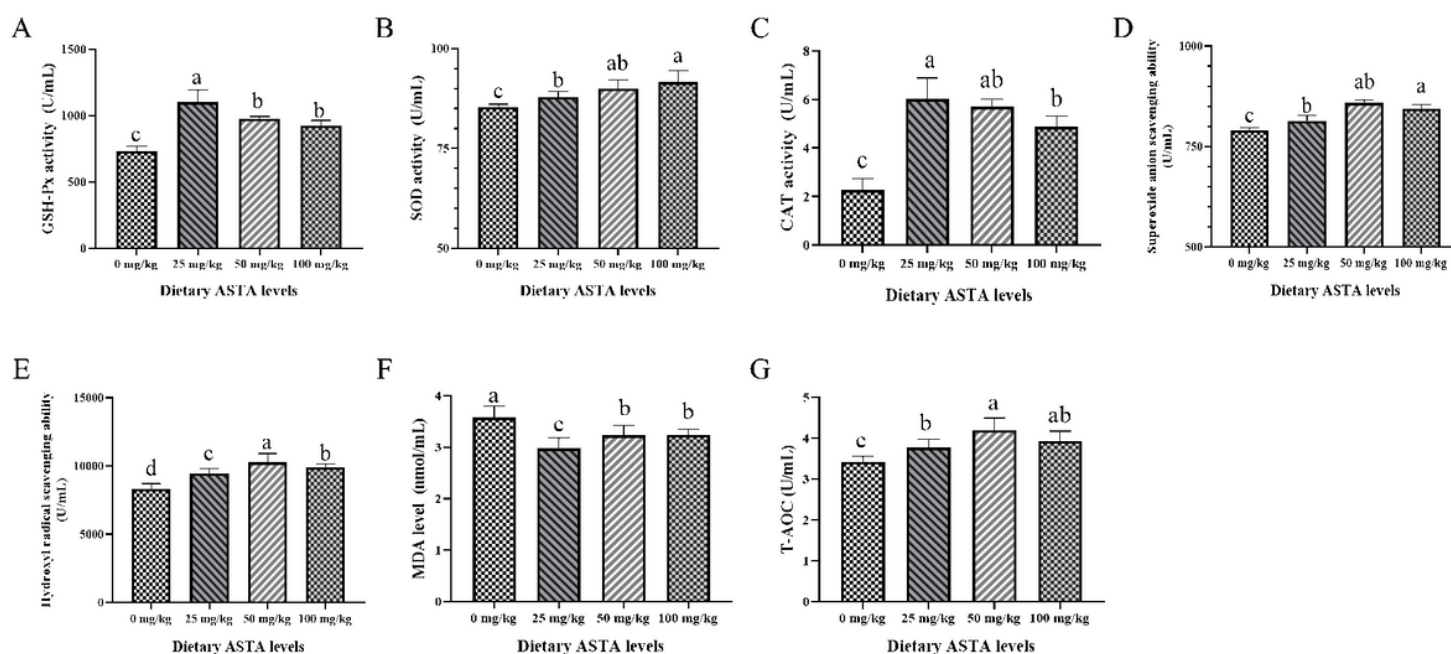


Figure 1

Effect of dietary natural astaxanthin (ASTA) supplementation on the antioxidant enzyme activity and free radicals in the plasma of aging layer breeder roosters. (A-C) Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) activity in the plasma. (D-E) Scavenging free radical abilities in the plasma. (F) Malondialdehyde (MDA) level in the plasma. (G) Total antioxidant capacity (T-AOC) in the plasma. The data represent the mean \pm standard deviation; $n=6$ in each group. a–d Means within a row with no common superscripts differ significantly ($P < 0.05$).

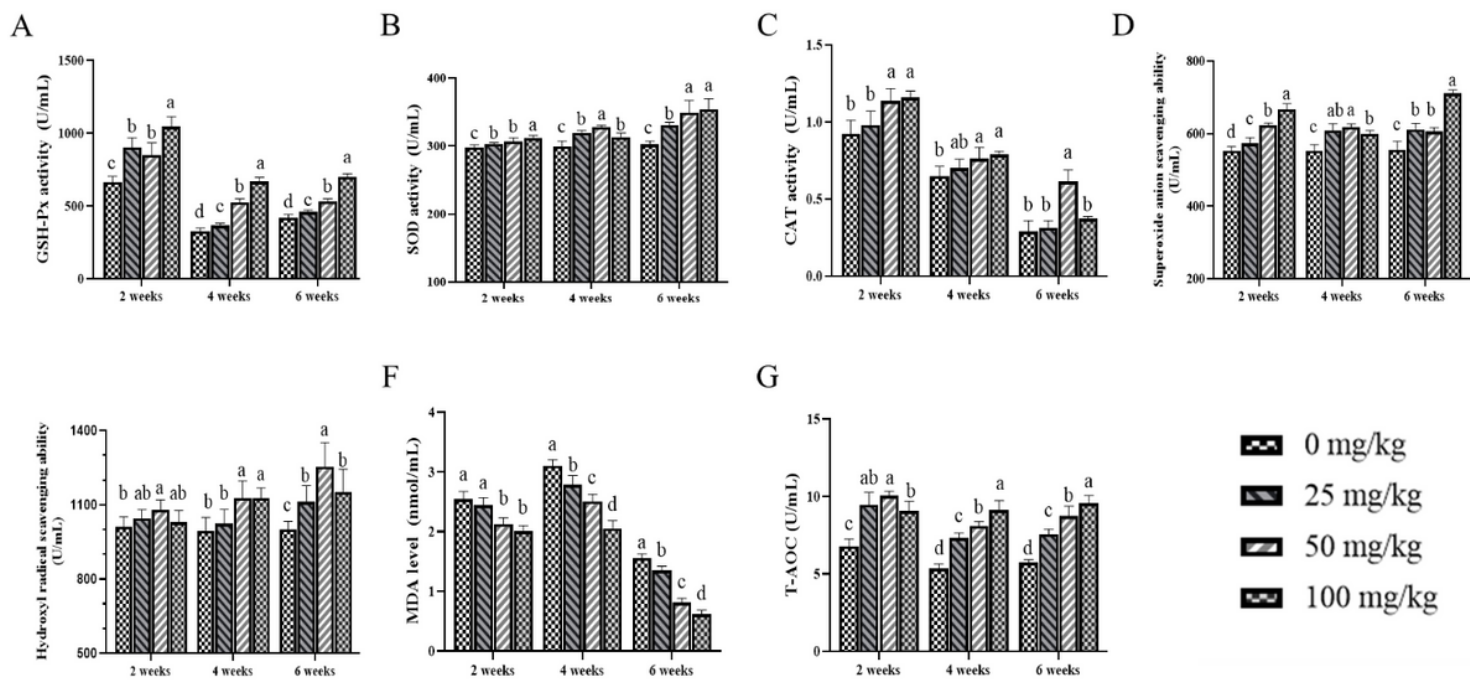


Figure 2

Effect of dietary natural astaxanthin (ASTA) supplementation on antioxidant enzyme activity and free radicals in the seminal plasma of aging layer breeder roosters. (A-C) Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) activity in the seminal plasma. (D-E) Scavenging free radical abilities in the seminal plasma. (F) Malondialdehyde (MDA) level in the seminal plasma. (G) Total antioxidant capacity (T-AOC) in the seminal plasma. The data represent the mean \pm standard deviation; $n=6$ in each group. a–d Means within a row with no common superscripts differ significantly ($P < 0.05$)

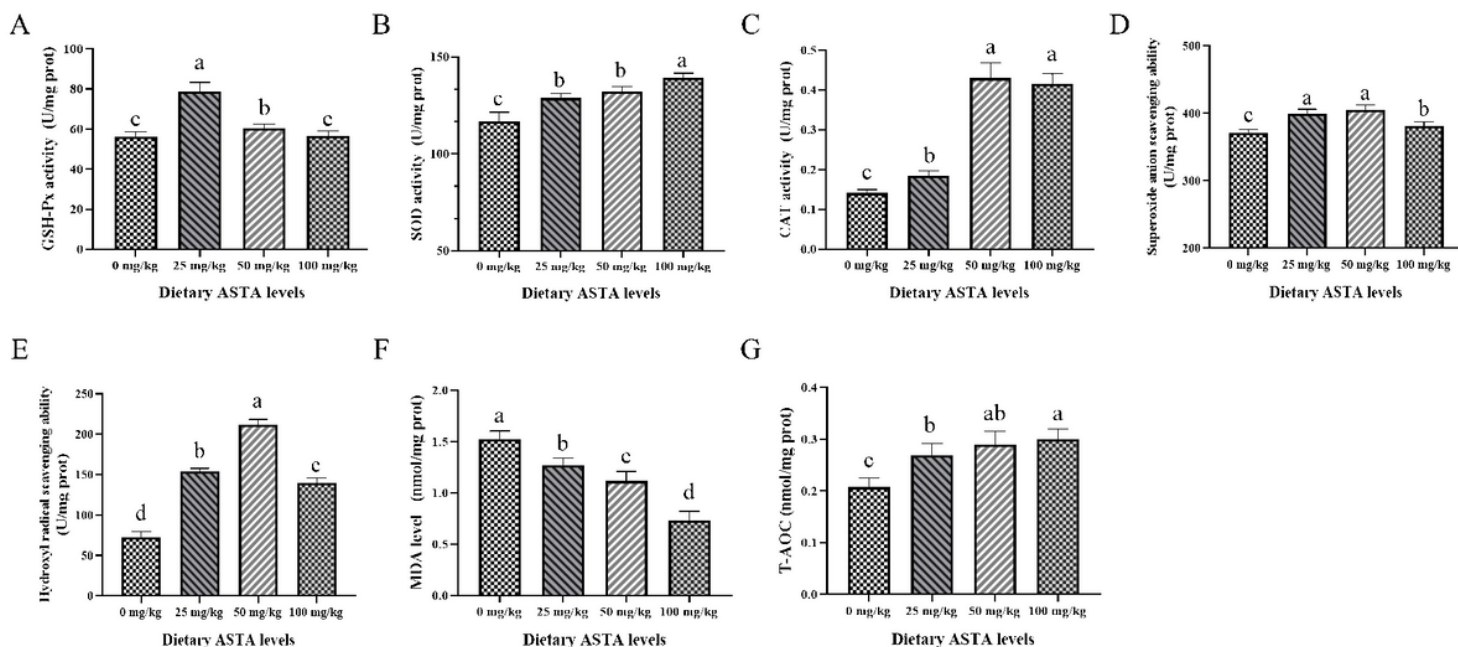


Figure 3

Effect of dietary natural astaxanthin (ASTA) supplementation on the antioxidant enzyme activity and free radicals in the testis of aging layer breeder roosters. (A-C) Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) activity in the testis. (D-E) Scavenging free radical abilities in the testis. (F) Malondialdehyde (MDA) level in the testis. (G) Total antioxidant

capacity (T-AOC) in the testis. The data represent the mean \pm standard deviation; $n=6$ in each group. a–d Means within a row with no common superscripts differ significantly ($P < 0.05$).

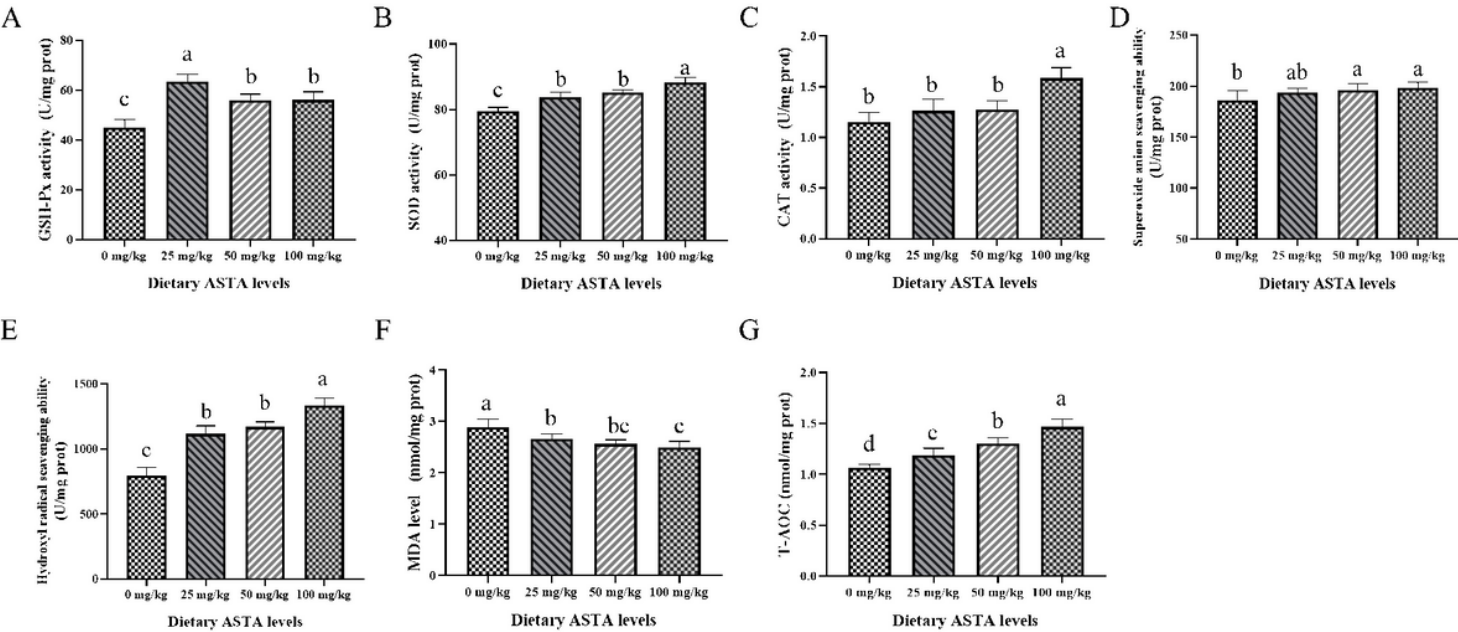


Figure 4

Effect of dietary natural astaxanthin (ASTA) supplementation on the antioxidant enzyme activity and free radicals in the liver of aging layer breeder roosters. (A-C) Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) activity in the liver. (D-E) Scavenging free radical abilities in the liver. (F) Malondialdehyde (MDA) level in the liver. (G) Total antioxidant capacity (T-AOC) in the liver. The data represent the mean \pm standard deviation; $n=6$ in each group. a–d Means within a row with no common superscripts differ significantly ($P < 0.05$).

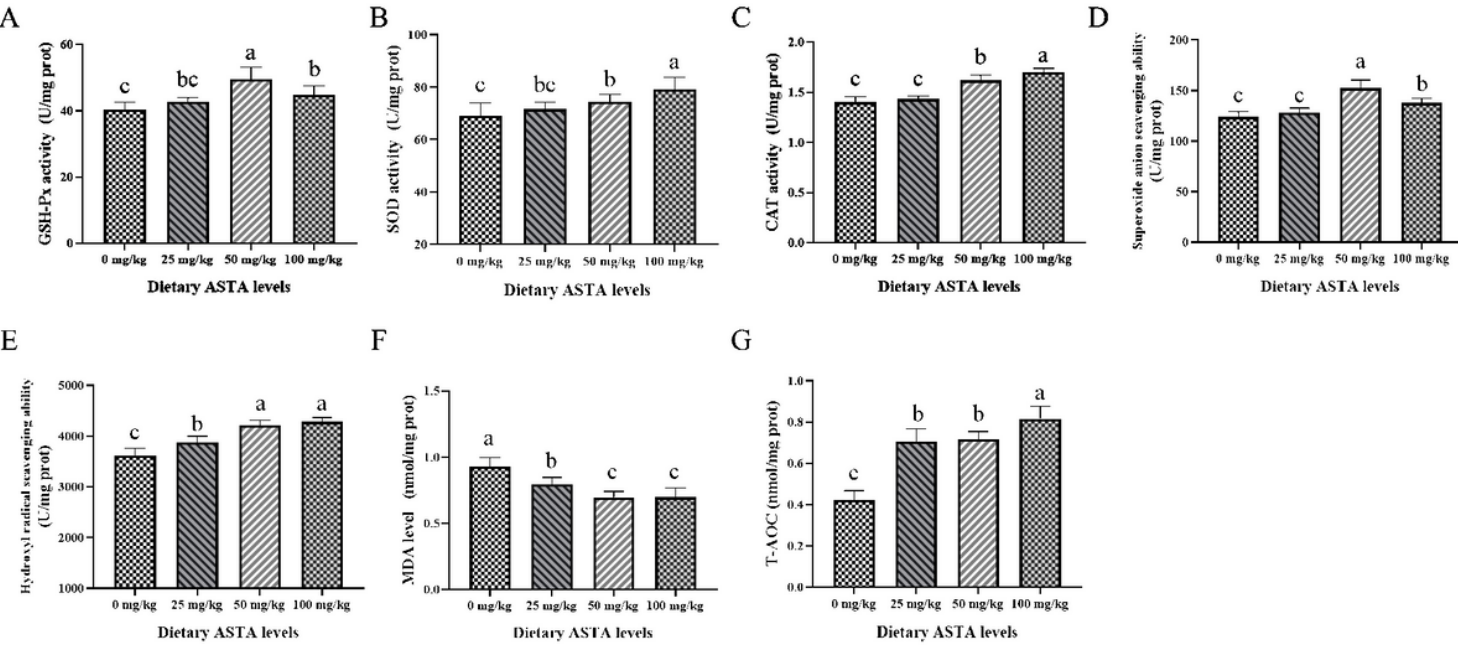


Figure 5

Effect of dietary natural astaxanthin (ASTA) supplementation on the antioxidant enzyme activity and free radicals in kidney of aging layer breeder roosters. (A-C) Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) activity in the kidney. (D-E) Scavenging free radicals abilities in the kidney. (F) Malondialdehyde (MDA) level in the kidney. (G) Total

antioxidant capacity (T-AOC) in the kidney. The data represent the mean \pm standard deviation; $n=6$ in each group. a–c Means within a row with no common superscripts differ significantly ($P < 0.05$).

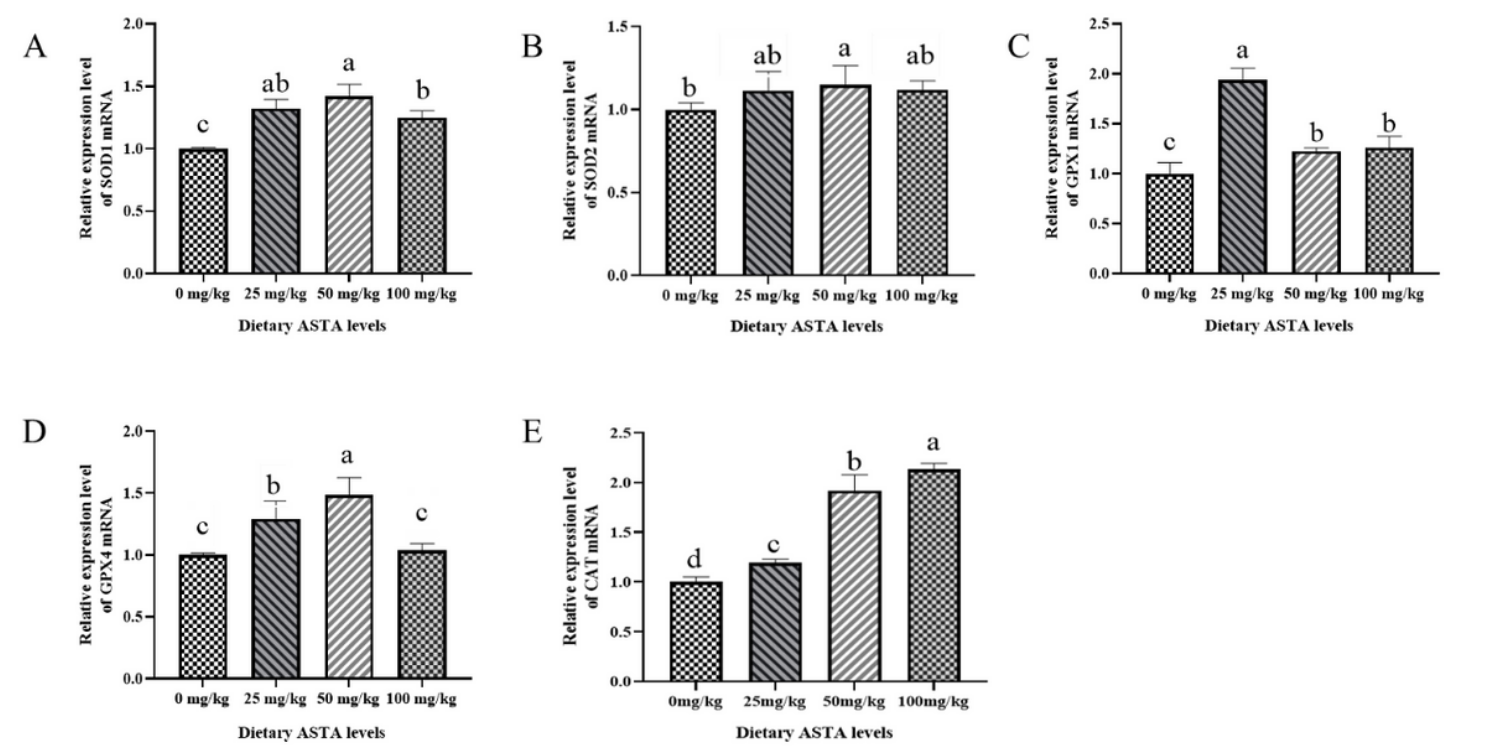


Figure 6

Effects of dietary natural astaxanthin (ASTA) supplementation on the mRNA expression of antioxidant enzymes, including Cu-Zn superoxide dismutase (SOD1) (A), Mn superoxide dismutase (SOD2) (B), glutathione peroxidase 1 (GPX1) (C), peroxidase 4 (GPX4) (D), and catalase (CAT) (E), relative to that of β -actin (ACTB). The values are expressed as the means \pm standard deviation of six birds per treatment. a–d Means within a row with no common superscripts differ significantly ($P < 0.05$).

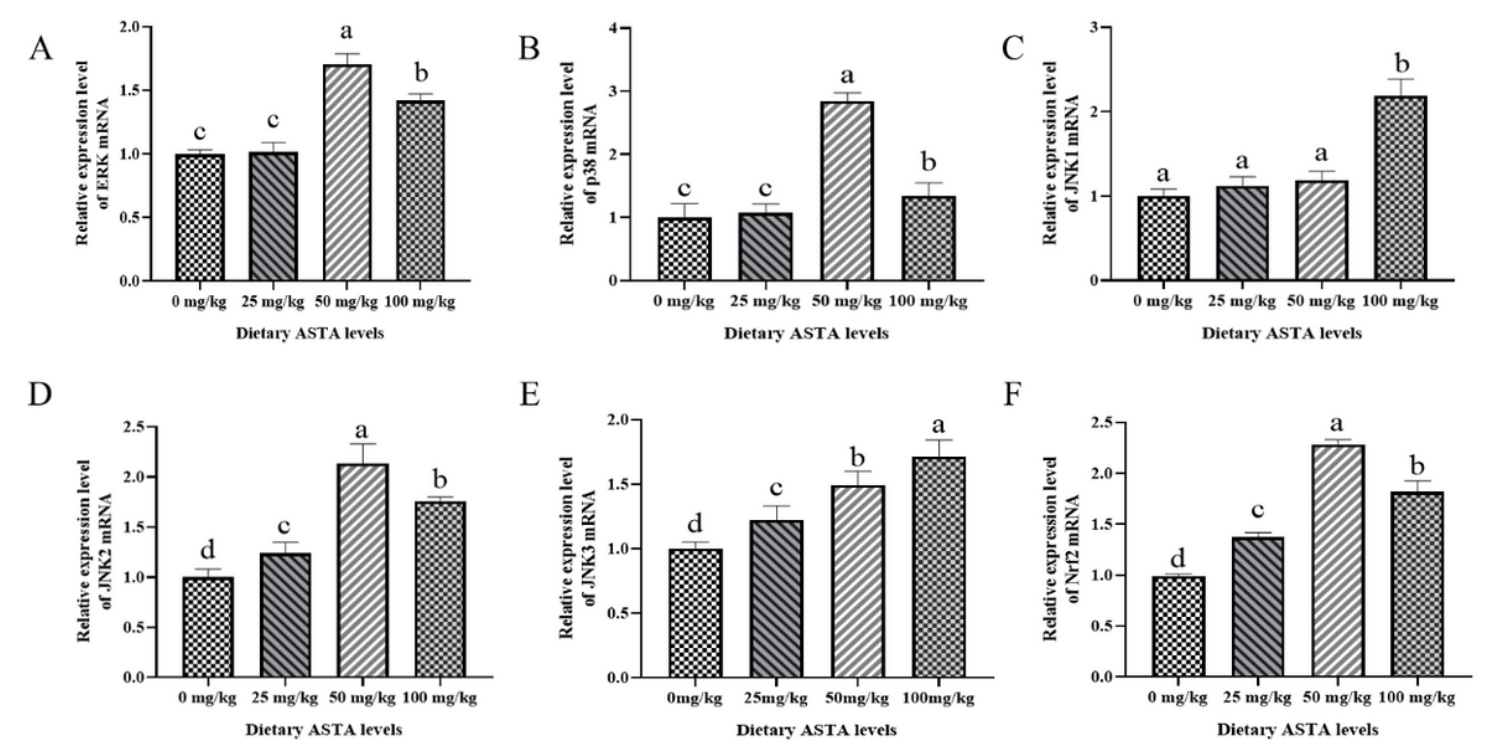


Figure 7

Effects of dietary natural astaxanthin (ASTA) supplementation on the mRNA expression of the mitogen-activated protein kinase/nuclear factor-erythroid 2-related factor 2 (MAPK/Nrf2) signaling pathway, including extracellular signal-regulated kinase (ERK) (A), p38 (B), c-Jun N-terminal kinase 1 (JNK1) (C), c-Jun N-terminal kinase 2 (JNK2) (D), c-Jun N-terminal kinase 3 (JNK3) (E), and Nrf2 (F) relative to that of β -actin (ACTB). The values are expressed as the means \pm standard deviation of six birds per treatment. a–d Means within a row with no common superscripts differ significantly ($P < 0.05$).

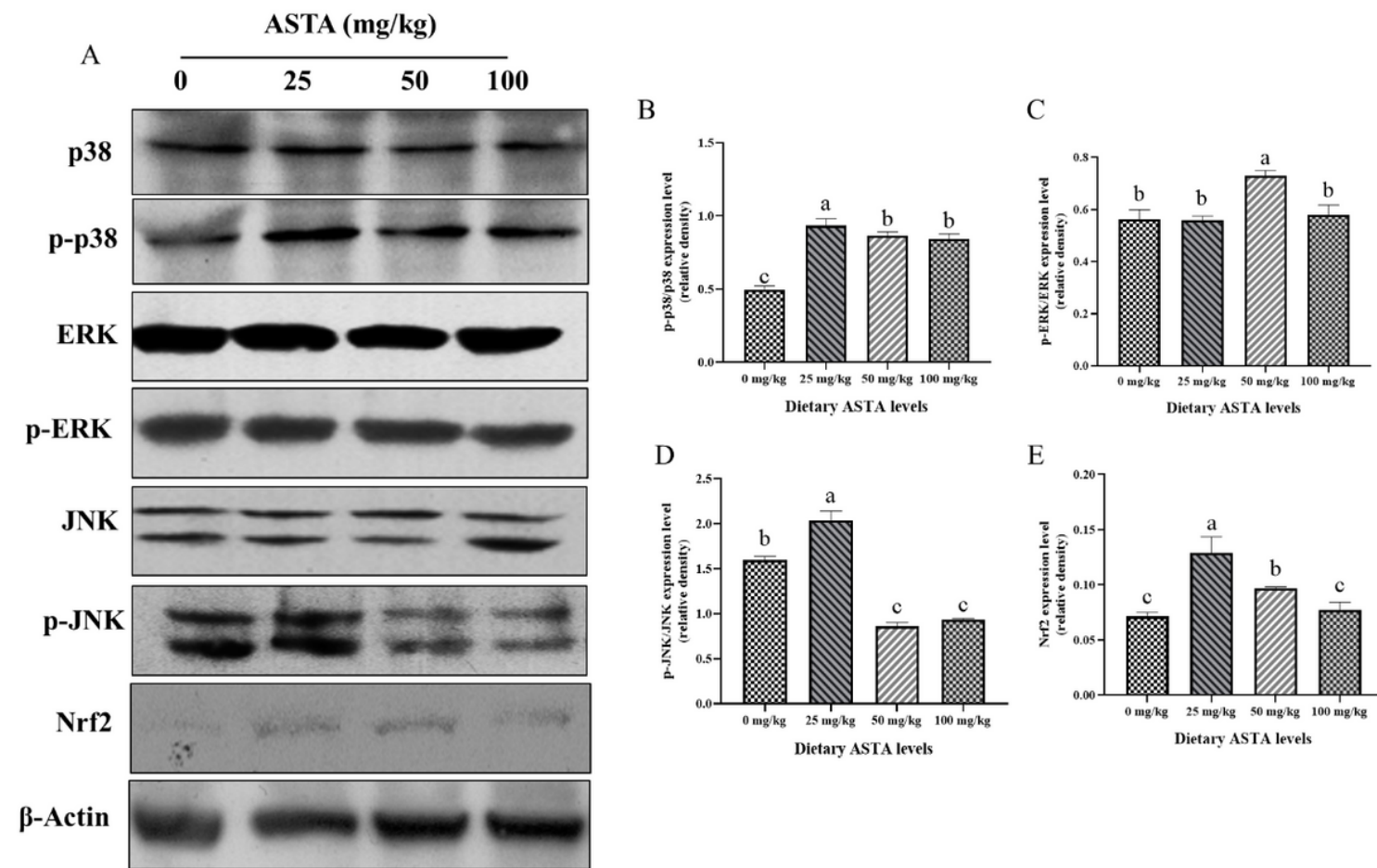


Figure 8

Effects of dietary natural astaxanthin (ASTA) on the expression of proteins related to the mitogen-activated protein kinase/nuclear factor-erythroid 2-related factor 2 (MAPK/Nrf2) pathway in the testis tissues of aging layer breeder roosters. The experimental groups included the control group (0 mg/kg) and ASTA groups (25-100 mg/kg). The results are expressed as the means \pm standard deviation ($n = 3$). a–c Means within a row with no common superscripts differ significantly ($P < 0.05$).

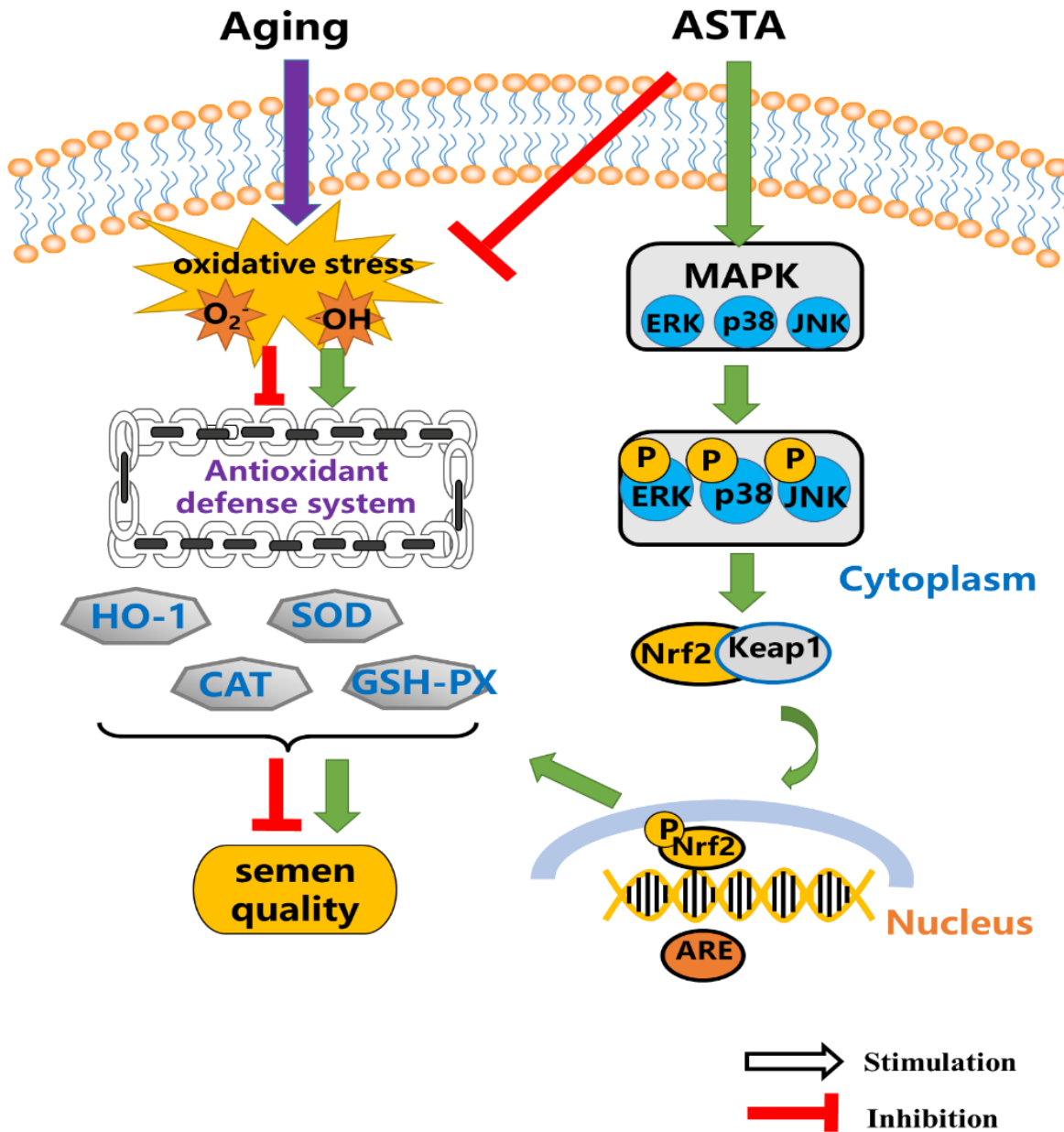


Figure 9

Schematic diagram summarizing the mechanisms by which natural astaxanthin (ASTA) promotes the antioxidant defense system in aging roosters. The antioxidant defense system is down-regulated in the natural aging process in roosters. ASTA attenuated the oxidative stress in the testis via the activation of the mitogen-activated protein kinase/nuclear factor-erythroid 2-related factor 2 (MAPK/Nrf2) pathway to attenuate the age-related sub-fertility in aging layer breeder roosters. ARE, antioxidant responsive element; CAT, catalase; GSH-Px, glutathione peroxidase; ERK, extracellular signal-related kinase; JNK, c-Jun N-terminal kinase; SOD, superoxide dismutase.