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Dietary supplementation with barley sprouts and D-aspartic acid improves reproductive hormone concentrations, testicular histology, antioxidant status, and mRNA expressions of apoptosis-related genes in aged broiler breeder roosters

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ABSTRACT

The objective was to determine effects of dietary supplementation of barley sprouts (BS) and/or D-aspartic acid (DA) on the reproductive potential of aged broiler roosters. Birds (n = 32, 50 wk old) were randomly allocated to receive dietary supplements of BS powder (2 % of basal diet), and DA (200 mg/kg BW), both, or neither, for 12 wk. Roosters were housed individually, with 14-h light/10-h dark, *ad libitum* feed and water, and euthanized after 12 wk. Mean (±SEM) total phenolic compounds and IC_{50} in methanol extract of sprouted barley were 302.8 ± 10.9 mg GAE/g and 600.8 ± 50.5 mg TE per 100 g, respectively. In weekly semen collections, sperm total and progressive motility, plasma membrane integrity, sperm concentration, and sperm production were higher ($P < 0.05$) in both the DA + BS and BS groups compared to the Control, but sperm abnormalities were unaffected. Feeding DA increased right, left, and combined testicular weights ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively) and, the testicular index ($P = 0.01$). Feeding BS increased seminiferous tubule diameter ($P < 0.01$), whereas BS + DA increased seminiferous epithelium thickness ($P < 0.01$). There were more spermatogonia ($P < 0.01$) and Leydig cells ($P < 0.05$) in BS-fed roosters but Sertoli cells were highest in BS + DA ($P < 0.01$). Serum MDA concentrations were lowest in BS ($P < 0.01$), whereas serum testosterone and LH were highest in DA ($P < 0.05$) and BS + DA ($P < 0.01$), respectively. Feeding BS reduced serum total cholesterol ($P < 0.05$) and increased serum HDL-cholesterol ($P < 0.01$), with decreases in serum LDL ($P < 0.01$) and the LDL/HDL ratio ($P < 0.01$) for BS + DA compared to Control. Relative expression of glutathione peroxidase mRNA was increased by BS ($P < 0.01$) or DA ($P < 0.05$), whereas relative mRNA expression of *SOD* was highest in BA ($P < 0.01$). Control roosters were highest for both BAX ($P < 0.01$) and the relative expression of the *BAX/BCL-2* ratio ($P < 0.01$), whereas BS + DA increased *BCL-2* ($P < 0.05$). In conclusion, feeding BS, and/or DA significantly improved reproductive potential in aged broiler roosters.

1. Introduction

At 50 wk of age, fertility declines rapidly in broiler breeder roosters, with decreases in sperm concentration, sexual behavior, and blood androgen concentrations [1]. As roosters age, testicular antioxidant systems are compromised, causing reproductive dysfunctions due to testicular apoptosis, impaired spermiation, and changes in serum biochemistry. Altered spermiation has been linked to Sertoli cell dysfunction reducing sperm production and fewer Leydig cells decreasing blood androgen concentrations [2].

Cereal sprouts are gaining attention as functional foods compared to dry grains, due to high nutritional values and health-promoting factors

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[3]. Sprouted barley (*Hardeum vulgare* L.) has health-promoting properties including antioxidant [4] and anti-inflammatory [3], effects, as well as improvements in blood chemistry [5]. Antioxidant properties of barley sprouts (BS) were attributed to phenolic compounds and bioactive flavonoids produced during sprouting and recovered by steeping [4]. In laying Japanese quail, BS increased rates of egg laying rate, fertilization, and hatching [6]. In addition, a micro-greens barley diet significantly reduced oxidative stress and reproductive disorders associated with type 2 diabetes mellitus and aflatoxicosis in rats [7].

D-aspartic acid (DA), an endogenous amino acid, increased serum concentrations of follicle-stimulating hormone, luteinizing hormone, and testosterone [8]. In addition, DA regulates androgen synthesis and release via the hypothalamic-pituitary-gonadal axis [9]. Treatment with DA enhanced StAR, P450scc, and 3BHSD mRNA expression in rat testes [10]. Improved sperm production occurred concurrently with DA accumulation in Leydig and Sertoli cells, and there were increases in testicular fluid flow, spermatogonia, elongated spermatids, and sperm [11]. Furthermore, DA enhanced sperm concentration, motility, and blood testosterone concentrations in humans [12] and rabbits [13]. Our objective was to determine the effects of dietary supplementation of BS and/or DA on the reproductive potential of aged broiler roosters.

2. Materials and methods

2.1. Materials

Unless otherwise specified, all chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

2.2. Sprouting and extraction

Sprouting was conducted as previously described [3] with slight modifications. Barley seeds (*Hordeum vulgare* L.) were washed and soaked for 24 h at 25 °C in darkness, with water changed at 2-h intervals. Thereafter, seeds were left to sprout in the dark for 2 d at 22–25 °C. After germination, seeds were spread over filter paper and allowed to grow at 60 % RH at 22–24 °C in the dark. Ten days after sprouting, sprouts were placed in a dryer at 40 °C until water content reached 10 %, then placed in sealed bags and stored at −20 °C.

Barley sprouts were extracted as described [14] with some modifications. In brief, 10 g of samples were macerated for 10 min with a pestle and mortar, and 10 % (weight/volume) of the extract was prepared with 70 % methanol. The extract was stirred for 24 h in a water bath at room temperature, filtered with Whatman filter paper (#1), and filtrate concentrated dry with a rotatable evaporator at 40 °C. Dried extracts were dissolved using 95 % methanol as a stock solution to obtain 50 mL, stored at 4 °C, and used to prepare working solutions.

2.3. Determination of total phenolic content and antioxidant activity of barley sprouts

Total phenolic content (TPC) was determined in a barley methanolic sprouted extract using the Folin-Ciocalteu [15] colorimetric procedure, with slight modifications. In brief, 0.5 mL of the extract was mixed with 2 mL of F–C reagent. After 5 min, 1.6 mL of Na_2CO_3 (7.5 %, w/v) was added and incubated at 45 °C for 45 min. A spectrophotometer (UV–visible, UV1800, Jinghua Instrument Co., Ltd., Shanghai, China) was used to measure absorbance at 760 nm against the blank solution containing 0.5 mL 95 % methanol, 2.5 mL, 10 % F–C reagent, and 2.5 mL of 7.5 % Na_2CO_3. Each sample was measured three times and expressed as milligrams of chlorogenic acid equivalents (CAEs) per gram of dried extract. The TPC was derived from a calibrated curve ($R^2 = 0.991$), using gallic acid as the standard (0.001–0.006 mg/mL), prepared by dissolving 10 g of BS in 100 mL of methanol (70 %, v/v) in an ultrasonic bath for 20 min, agitated with a laboratory shaker at 200 rpm in the dark for 2 h at ambient temperature, and then filtered with Whatman paper (#1).

Antioxidant activity was measured in the barley sprouted methanolic extract using a conventional DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay [16], with minor modifications. Absorbance was measured using a spectrophotometer at 515 nm. Free radical scavenging activity was calculated as follows:

$$\text{DPPA scavenge activity } \% = [(\text{Ac} - \text{As sample})/\ \text{Ac blank}] \times 100$$

where Ac and As are absorbances of the control and sample, respectively. The antioxidant activity of the extract was represented as IC_{50}, the concentration of the sample needed to scavenge 50 % of the DPPH free radical, calculated based on non-linear regression analysis of the scavenging activity of DPPH radicals against various sample concentrations. The IC_{50} values were reported as mg sample (DM)/mL solution of DPPH [17].

2.4. Birds and experimental design

All trial protocols were approved by the Animal Care and Use Committee of the Gorgan University of Agricultural Sciences Natural Resources, Gorgan, Iran (Protocol 1398.165943/388). Broiler breeder roosters, Hubbard M77, (n = 32, 50 wk old) were randomly assigned into 4 treatments with 8 replications of individual birds in each treatment as a 2 × 2 factorial arrangement of treatments in a completely randomized design. The first factor was a powder of barley sprouts (BS), either 0 or 2 % of the basal diet, and the second factor was D-aspartate acid (DA), either 0 or 200 mg/kg BW for 12 wk. The doses used were based on reports by Abouelezz et al. (BS) [6] and Ansari et al. (DA) [18]. Roosters were kept in individual cages, with a 14-h light and 10-h dark regime. All roosters were fed a standard diet (Table 1), with feed and fresh water available *ad libitum*. Semen was collected and assessed

Table 1
Ingredients and chemical composition of the standard broiler breeder rooster diet.

Ingredient	Content (%)
Corn	62.1
Soybean meal	11.2
Wheat barn	22.97
Dicalcium phosphate	0.87
Calcium carbonate	1.55
Sodium chloride	0.22
DL-Met	0.09
L-Lys	0.1
Soybean Oil	0.3
Vitamin premix[a]	0.3
Trace mineral premix[b]	0.3
Digestible amino acids	
Lysine	0.5
Methionine	0.3
Leucine	0.53
Methionine and cysteine	0.5
Tryptophan	0.12
Arginine	0.65
Isoleucine	0.42
Valine	0.5
Threonine	0.39
Chemical Composition	
ME (Kcal/kg)	2650
CP	12.5
Ca	0.9
Available P	0.4
Na	0.17
Cl	0.16
K	0.6

[a] Supplied per kilogram of diet: vitamin A 15000 IU, vitamin E 100 IU, vitamin K_3 5 mg, vitamin B_{12} 0.03 mg, vitamin D 3000 IU, riboflavin 10 mg, niacin 60 mg, pantothenic acid 15 mg, folic acid 2 mg.

[b] Supplied per kilogram of diet: Fe (60 mg), Mn (60 mg), Zn (100 mg), I (2 mg), Se (0.4 mg).

weekly during the first 10 wk of the experiment.

2.5. Semen sampling and assessment

Semen was collected using abdominal massage, as described by Burrows and Quinn [19]. To nullify the individual effects, ejaculates from each bird in each replicate (n = 8) were pooled and evaluated as a single sample [20]. Seminal volume was measured using a graduated collecting tube. Sperm concentration was determined after dilution of the semen sample (1:200 with distilled water), and then a droplet of diluted semen was placed on the Neubauer hemocytometer. Total sperm production (total sperm per ejaculate $\times 10^9$) was calculated by multiplying ejaculate volume and sperm concentration.

To determine sperm total and progressive motility, pooled semen samples were diluted with 2.9 % sodium citrate (1:20), a drop of diluted semen was loaded onto a pre-warmed (37 °C) microscope slide and covered with a coverslip (on a 37 °C warm stage), and then 10 microscopic fields were observed by one person using a phase contrast microscope (Carl Zeiss, Oberkochen, Germany; 400× magnification) to evaluate sperm motility on at least 200 sperm per slide. Total motility was expressed as a percentage of motile sperm whereas progressive motility was the percentage of sperm with progressively moderate or quick movement [20,21].

Eosin-nigrosin staining was used to determine sperm viability and abnormalities. Unstained sperm were considered live and those with any kind of morphologic abnormality including detached heads, abaxial heads, malformed heads, bent tails, coiled tails, double tails, or protoplasmic droplets considered abnormal [22]. Abnormal sperm were expressed as percentages of the total number of sperm evaluated.

To evaluate sperm plasma membrane integrity, a hypo-osmotic swelling test (HOST) was performed, as described [23], with slight modifications. This assay was conducted by mixing 10 μL of pooled semen with 200 μL hypoosmotic sodium citrate solution (100 mOsm). After 30 min incubation, membrane integrity of 200 sperm was assessed at 400× magnification with a phase-contrast microscope (Carl Zeiss). Sperm with swollen or non-swollen tails were recorded as having membrane integrity or non-integrity, respectively.

2.6. Blood sampling and measurement of hormones and lipids

At 62 wk of age, roosters were euthanized by cervical dislocation. Blood samples (5 mL) were obtained from the brachial vein, put into a 10 mL Vacutainer tube (no anticoagulant), kept on ice, protected from light to prevent oxidation, allowed to clot, and centrifuged at 1500×*g* at 4 °C for 10 min. The serum was removed and stored at −20 °C.

A clinical chemistry analyzer (AU2700, Olympus, Tokyo, Japan) was used to determine blood serum concentrations of total cholesterol, triglycerides, and high-density lipoprotein cholesterol (HDL-chol). Low-density lipoprotein cholesterol (LDL-chol) was calculated as:

LDL-chol = total cholesterol - [HDL-chol+(triglyceride/5)] [24].

Serum concentrations of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were measured with ELISA (Crystal Day Biotech Co, Shanghai, China), according to the manufacturer's instructions. All assays were performed in 96-well plates and absorbance was measured at 450 nm. Testosterone was measured using ELISA (Monobind Inc., Costa Mesa, CA, USA). The coefficient of variation and sensitivity of the testosterone assay were 6.1 % and 0.0576 ng/mL, respectively.

2.7. Tissue processing and histological analyses

Both testes were excised and weighed, and the testicular index (testicular weight/body weight) was calculated [1]. Each right testis was cut into two parts; one was frozen in liquid nitrogen to prevent RNA degradation and subjected to total RNA extraction and real-time polymerase chain reaction (RT-PCR). The remainder was fixed in neutral buffered formalin (10 %), stored at −4 °C, embedded in paraffin, sectioned (5–6 μm), and stained with hematoxylin-eosin [1]. Morphological data were generated from 20 images randomly selected from four cross-sections of each testis using an optical microscope (Zeiss) equipped with an eyepiece camera (Labomed Inc., Los Angeles, CA, USA) and analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA). The diameter and number of seminiferous tubules and thickness of the seminiferous epithelium were determined from 20 randomly selected seminiferous tubule segments [25]. Leydig and Sertoli cells and blood vessels were also enumerated. All histological assessments were performed "in the blind" regarding the treatment group.

2.8. Testicular oxidative status

Malondialdehyde (MDA) concentration was determined as described [26] and expressed in nmol per mg protein of testicular tissue. Furthermore, total protein content was estimated by a bicinchoninic acid assay using a Takara kit [27].

2.9. RNA extraction and real-time polymerase chain reaction

Frozen testicular portions were used for mRNA extraction. Total RNA was extracted using the RNeasy Mini Kit (Cat. No. 74104; Qiagen, GmbH, Germany), according to the manufacturer's protocol. Using the QuantiTec Reverse Transcription Kit (Cat. No. 205311; Qiagen, GmbH, Germany), complementary DNA (cDNA) was synthesized with reaction conditions of 42.8 °C for 30 min and 95.8 °C for 3 min. Primers for real-time polymerase chain reaction (RT-PCR) were designed using Primer 3 software [28] and sequenced for superoxide dismutase (*SOD*), glutathione peroxidase (*GSH-P$_X$*), B-cell lymphoma-2 (*BCL-2*), and Bcl-2-associated X protein (*BAX*), with β-actin as internal control (Table 2). Quantification of all transcripts used the QuantiFast SYBR Green PCR Kit (Cat. No. 204054; Qiagen) in a 20 μL reaction volume containing 1 μL single-strand cDNA, 10 μL of master mix, 0.5 μL of either forward and reverse primers and 8 μL of distilled H_2O in 20 μL, using Rotor-Gene 6000 Real-Time PCR software (Corbett Research, Sydney, Australia). The PCR program consisted of a 5-min activation step at 95

Table 2
Details of primer sequences used for quantitative real-time PCR of rooster testicular tissue.

Gene	Sequence (5′–3′)	Product length (bp)	Accession no.
SOD[a]	Forward: TTGTCTGATGGAGATCATGGCTTC Reverse: TGCTTGCCTTCAGGATTAAAGTGAG	98	NM_205064.1
GSH-P$_X$[b]	Forward: GCTGTTCGCCTTCCTGAGAG Reverse: GTTCCAGGAGACGTCGTTGC	118	NM_001277853.1
BCL-2[c]	Forward: GATGACCGAGTACCTGAACC Reverse: CAGGAGAAATCGAACAAAGGC	114	NM_205339
BAX[d]	Forward: TCCTCATCGCCATGCTCAT Reverse: CCTTGGTCTGGAAGCAGAAGA	69	XM422067
β-actin	Forward: AGCCAACAGAGAGAAGATGACAC Reverse: CATCACCAGAGTCCATCACAATA	134	L08165.1

[a] Superoxide dismutase.
[b] Glutathione peroxidase.
[c] B-cell lymphoma-2.
[d] Bcl-2-associated protein.

°C, followed by 40 cycles of 15 s at 95 °C, and 40 s at 60 °C. At the end of each PCR, for all genes, a melting curve analysis was performed at the rate of 0.1 °C/s to verify product specificity. The efficiency of the tests was >95 % and the standard curve R^2 was >0.999. Normalized values were calculated as a ratio of target gene mRNA to β-actin mRNA quantities. The qPCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method [29].

2.10. Statistical analyses

The Shapiro-Wilk and arc sine tests were used to test the data for the normality and for transformation of percentage data when appropriate, respectively. Data were analyzed as a 2 × 2 factorial arrangement using the GLM (for one-time data such as histology, blood samples, and gene expression) and MIXED (for repeated data such as weekly semen) procedures of SAS 9.2 (SAS Institute, Cary, NC, USA), with a model that included the main effects of BS and DA, as well as their interaction. When there was a significant main effect or interaction, Duncan's multiple comparisons test was used to separate means. Differences were considered significant at $P < 0.05$ and extremely significant at $P < 0.01$ levels, although probability values up to $0.05 \leq P < 0.01$ are reported in the text if data indicated a trend. Moreover, data for mRNA expression were normalized before using a relative expression software tool [30] for statistical analysis.

3. Results

3.1. Total phenolic content and antioxidant activity

The total phenolic content (TPC) and antioxidant properties of the methanol extract of sprouted barley are in Table 3. Using gallic acid as a standard, TPC in sprouted barley extractions was determined by the Folin-Ciocalteu procedure. To construct a calibration curve, absorbance values from various concentrations of gallic acid were used. Based on this regression equation ($Y = 0.0108x$; $R^2 = 0.992$), the TPC was calculated as mg of gallic acid equivalents (GAE) per gram of dry weight (mg/g) per sample. Accordingly, the methanol extract of sprouted barley had a TPC value of 302.8 ± 10.9 mg GAE/g. There are several phenolic compounds in sprouted barley, but vanillic acid, catechin, and epicatechin are the most abundant. A DPPH free radical scavenging assay was used to determine the antioxidant activity of sprouted barley methanol extract, with the IC_{50} determined by measuring the amount of sample required to scavenge 50 % of DPPH free radicals present in a given concentration. The IC_{50} value was 600.77 ± 50.53 mg TE per 100 g.

3.2. Sperm quality

The effects of dietary treatments and their interactions on various sperm end points are in Table 4. For most sperm end points (excluding sperm abnormalities and ejaculate volume), there was a significant effect of treatment, time or their interaction. Both BS and BS + DA treatments had higher ($P < 0.01$) total and progressive motility compared to the Control and DA groups. Furthermore, the combination of BS + DA resulted in the greatest plasma membrane integrity, sperm concentration, and sperm production ($P < 0.01$).

Table 3
Mean ± SD (n = 3) characteristics of barley sprouts (BS) powder.

Compound[a]	Concentration in BS
TPC	302.8 ± 10.9
Protocatechuic acid	5.8 ± 0.2
Vanillic acid	83.8 ± 3.2
Catechin	61.3 ± 2.7
Epicatechin	47.8 ± 2.0
Chlorogenic acid	20.3 ± 0.8
Gallic acid	43.2 ± 1.1
Ferulic acid	14.5 ± 0.4
Sinapic acid	19.5 ± 0.8
Myricetin	2.6 ± 0.1
Quercetin	0.7 ± 0.03
Kaempferol	3.3 ± 0.08
IC_{50}^{DPPH}	600.8 ± 50.5

[a] Results are presented in mg 100 g^{-1}, where TPC in mg GAE 100 g^{-1}, IC_{50}^{DPPH} in mg TE 100 g^{-1}.

3.3. Body weight and testicular end points

Body weight and testicular end points are in Tables 5 and 6. Body weight was not significantly different among groups. Dietary DA supplementation increased right, left, and combined testicular weights ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively) and testicular index ($P < 0.01$). Feeding BS increased seminiferous tubule diameter ($P < 0.01$) and BS + DA increased seminiferous epithelium thickness ($P < 0.01$; Fig. 1). There were more spermatogonia ($P < 0.01$) and Leydig cells ($P < 0.05$) in BS-fed roosters. Moreover, the number of Sertoli cells was increased ($P < 0.01$) by feeding BS + DA. Blood vessels were not significantly affected by dietary supplementation.

3.4. MDA peroxidation index, blood serum hormones, and cholesterol profiles

The effects of dietary BS and DA supplementation on testicular MDA and serum chemistry are in Table 7. Feeding BS reduced ($P < 0.01$) MDA concentrations whereas DA increased serum testosterone concentrations ($P < 0.01$). Serum LH concentrations were increased by an interaction ($P < 0.01$), whereas FSH concentrations were not affected. Feeding BS reduced serum concentrations of total cholesterol ($P < 0.05$) and increased serum concentrations of HDL-cholesterol ($P < 0.01$). However, BS + DA decreased serum LDL ($P < 0.05$) and the LDL/HDL ratio ($P < 0.01$).

3.5. Gene expression

Testicular expressions of mRNA for glutathione peroxidase (*GSH-P_X*), superoxide dismutase (*SOD*), *BAX*, and *BCL-2* are shown in Fig. 2. There was up-regulation of glutathione peroxidase relative expression in roosters fed BS ($P < 0.01$) or DA ($P < 0.05$). Control roosters had the lowest relative mRNA expression of *SOD* ($P < 0.01$) and the highest lowest relative mRNA expression of *BAX* ($P < 0.01$). Expression of *BCL-2* expression was highest in BS + DA ($P < 0.05$) and the relative expression of *BAX/BCL-2* ratio was highest in the Control and DA groups ($P < 0.01$).

4. Discussion

After peak production (37 wk of age), broiler breeder roosters have decreases in serum gonadotropin concentrations and the testosterone to estradiol ratio, and in their testes, histological changes, and reduced antioxidant capacity [1,31]. In this study, feeding DA, BS, or both significantly improved plasma LH and testosterone concentrations, blood lipid profile, and in their testes, morphological end points, and the antioxidant system. Furthermore, qualitative histological findings for testicular tissue confirmed quantitative data.

Cereal sprouts have received increasing attention as functional foods due to their high nutritional value and health-promoting potential, particularly flavonoids and phenolic compounds [3], with the antioxidant potential of sprouted barley attributed to phenolic compounds produced during soaking and sprouting [32]. In this study, the TPC value of the methanol extract of sprouted barley (302.8 ± 10.9 mg GAE/100 g) was in accordance with a TPC value of 367.7 GAE/100 g for sprouted barley [33]. Similarly, in another study, the TPC value of the methanol extract of barley sprout was 381.7 ± 7.4 mg of GAE/100 g [34]. However, our results differed from Liu et al. [35] who reported 450 mg of GAE/100g. Differences could arise from variations in genetic

Table 4
The effects of experimental treatments on sperm end points in aged roosters.

Treatments	Sperm concentration (× 10^9 cells mL^{-1})	Ejaculate volume (mL)	Total sperm per ejaculate (× 10^9)	Progressive motility (%)	Total motility (%)	Plasma membrane integrity (%)	Abnormal sperm (%)
BS (%)							
0	3.33[b]	0.367	1.22[b]	70.94[b]	81.84[b]	82.27[b]	4.60
2	3.61[a]	0.373	1.35[a]	77.95[a]	88.31[a]	85.83[a]	4.59
Pooled SEM	0.02	0.02	0.09	0.18	0.20	0.25	0.03
DA (mg/kg BW)							
0	3.31[b]	0.367	1.22[b]	73.92[b]	84.57[b]	81.85[b]	4.59
200	3.63[a]	0.372	1.35[a]	74.97[a]	85.58[a]	86.24[a]	4.60
Pooled SEM	0.02	0.02	0.09	0.18	0.16	0.24	0.03
BS × DA							
0 × 0	3.09[c]	0.365	1.13[c]	70.85[c]	81.33[b]	78.41[b]	4.59
2 × 0	3.52[b]	0.370	1.31[b]	76.99[b]	87.81[a]	85.29[a]	4.59
0 × 200	3.57[b]	0.369	1.30[b]	71.04[c]	82.35[b]	86.12[a]	4.60
2 × 200	3.69[a]	0.375	1.39[a]	78.90[a]	88.82[a]	86.37[a]	4.60
Pooled SEM	0.03	0.03	0.01	0.30	0.32	0.41	0.05
P-value							
BS	0.001	0.069	0.001	0.001	0.001	0.001	0.580
DA	0.001	0.120	0.001	0.001	0.001	0.001	0.084
BS × DA	0.001	0.875	0.006	0.012	0.049	0.001	0.832
time	0.001	0.001	0.001	0.001	0.001	0.001	0.001
BS × time	0.007	0.490	0.003	0.001	0.001	0.001	0.024
DA × time	0.002	0.832	0.006	0.001	0.007	0.001	0.021
BS × DA × time	0.007	0.960	0.056	0.001	0.008	0.009	0.183

BS, barley sprout powder and DA, D-aspartic acid.
[a-c]Within a column and cluster (between horizontal lines), means without a common superscript differed ($P < 0.05$).

Table 5
The effects of experimental treatments on body weight and testicular weights and indices in aged roosters.

Treatments	Body weight (g)	Right testis weight (g)	Left testis weight (g)	CTW[a] (g)	Testicular index[b] (g/kg)
BS (%)					
0	4843	20.12	21.12	41.25	8.51
2	4874	20.50	22.37	42.87	8.79
Pooled SEM	11.23	0.56	0.64	0.83	0.16
DA (mg/kg BW)					
0	4865	19.25[b]	20.50[b]	39.75[b]	8.18[b]
200	4861	21.37[a]	23.00[a]	44.73[a]	9.12[a]
Pooled SEM	11.23	0.56	0.64	0.83	0.16
BS × DA					
0 × 0	4849	19.25[b]	20.00[b]	39.25[c]	8.09[c]
2 × 0	4863	19.25[b]	21.00[ab]	40.25[bc]	8.27[bc]
0 × 200	4838	21.00[a]	22.25[ab]	43.25[ab]	8.94[ab]
2 × 200	4885	21.75[a]	23.75[a]	45.50[a]	9.31[a]
Pooled SEM	15.88	0.79	0.90	1.18	0.23
P-value					
BS	0.079	0.645	0.193	0.194	0.270
DA	0.735	0.020	0.017	0.002	0.001
BS × DA	0.341	0.045	0.047	0.007	0.008

a-c: Within a column and cluster (between horizontal lines), means without a common superscript differed ($P < 0.05$).
[a] Combined testis weight (g) = left testis weight + right testis weight.
[b] Calculated as testis weight (g)/body weight (kg). BS, barley sprout powder and DA, D-aspartic acid.

backgrounds, germination time, environmental factors, agronomic practices, and polarity of solvents used for extraction.

The sample concentration that decreased the initial DPPH concentration by 50 % is defined as the IC_{50} and was used to calculate the antioxidant capacity of the extract [36]. The value of IC_{50} in the current study was 600.8 ± 50.5 mg TE/100 g DW, consistent with 666 ± 62.6 mg TE/100 g DW of buckwheat sprouts [37], but quite lower than 786.7 ± 9.3 TE/100 g DW [34] and higher than 559.7 TE/100 g DW [33].

Dietary supplementation of BS, DA, or both significantly increased total (except DA) and progressive (except DA) sperm motility and improved plasma membrane integrity. This apparent contradiction could be attributed to the basal diet, breed, age, and breeding condition. However, neither BS, DA nor their combination affected sperm morphology in aged roosters. Data on sperm motility contrasted with Ansari et al. [18], who reported greater sperm motility in aged roosters fed a diet supplemented with 100–300 mg/kg/d of DA. The present study's plasma membrane integrity improvements may have been mediated by NMDA-R (*N*-methyl d aspartic acid receptor), which is permeable to extracellular Ca^{2+}. Calcium and HCO_3 anion stimulate flagellum strokes through a pathway dependent on adenylyl cyclase and protein kinase A [38,39]. In this study, dietary inclusion of BS, DA, or both significantly increased sperm concentration. In line with sperm concentration, a similar result was observed by Ansari et al. [18], who fed aged roosters a diet supplemented with various DA concentrations (100–300 mg/kg/day).

Adding DA to the basal diet of broiler roosters increased blood testosterone concentrations, total number and motility of sperm, and fertilization rate [18], whereas in rats, it increased serum concentrations of FSH, LH, and testosterone [18,40]. DA increased the expression of enzymes involved in testosterone production by triggering LH release from the anterior pituitary, thereby increasing testosterone. The interaction between LH and its receptors has both acute and chronic effects on Leydig cells; the former is the formation of cAMP and the stimulation of cholesterol transfer into mitochondria, whereas the latter increases steroidogenic enzymes [41]. Greater testosterone production promotes expansion of the generative epithelium [42], consistent with the greater diameter of seminiferous tubules and a thicker seminiferous epithelium in this study. In addition, plasma testosterone concentrations and the number and activity of Sertoli cells are correlated [43]. Sperm retention in seminiferous tubules reduces sperm concentrations in older roosters [31]. Furthermore, as testosterone has a crucial role in the Sertoli cell-spermatid junctional complex, increasing testosterone concentrations in this study may also have increased sperm concentration, preventing germ cell phagocytosis in the absence of testosterone [44].

Table 6
The effects of experimental treatments on the mean testicular histological end points in aged roosters.

Treatments	STD[a] (μm)	SET[b] (μm)	No. spermatogonia	No. Leydig cells	No. Sertoli cells	No. blood vessels
BS (%)						
0	237.78[b]	78.81[b]	201.33[b]	22.25[a]	19.00[b]	1.18
2	298.56[a]	96.15[a]	214.56[a]	19.37[b]	22.62[a]	1.29
Pooled SEM	3.96	1.01	1.64	0.80	0.34	0.06
DA (mg/kg BW)						
0	265.09	87.34	207.13	20.25	20.75	1.19
200	271.26	87.62	208.76	21.37	20.78	1.27
Pooled SEM	3.96	1.01	1.64	0.80	0.34	0.06
BS × DA						
0 × 0	234.17[b]	76.38[c]	194.72[c]	18.75[b]	19.00[b]	1.15
2 × 0	296.00[a]	98.31[a]	219.54[a]	21.75[ab]	22.50[a]	1.24
0 × 200	241.38[b]	81.24[b]	207.95[b]	20.00[ab]	19.00[b]	1.21
2 × 200	301.13[a]	94.00[a]	209.58[b]	22.75[a]	22.75[a]	1.34
Pooled SEM	5.60	1.41	2.32	1.13	0.49	0.09
P-value						
BS	0.001	0.001	0.001	0.026	0.001	0.246
DA	0.292	0.847	0.804	0.342	0.494	0.413
BS × DA	0.008	0.007	0.008	0.009	0.003	0.855

a-c: Within a column and cluster (between horizontal lines), means without a common superscript differed ($P < 0.05$).
[a] Seminiferous tubule diameter.
[b] Seminiferous epithelium thickness. BS, barley sprout powder and DA, D-aspartic acid.

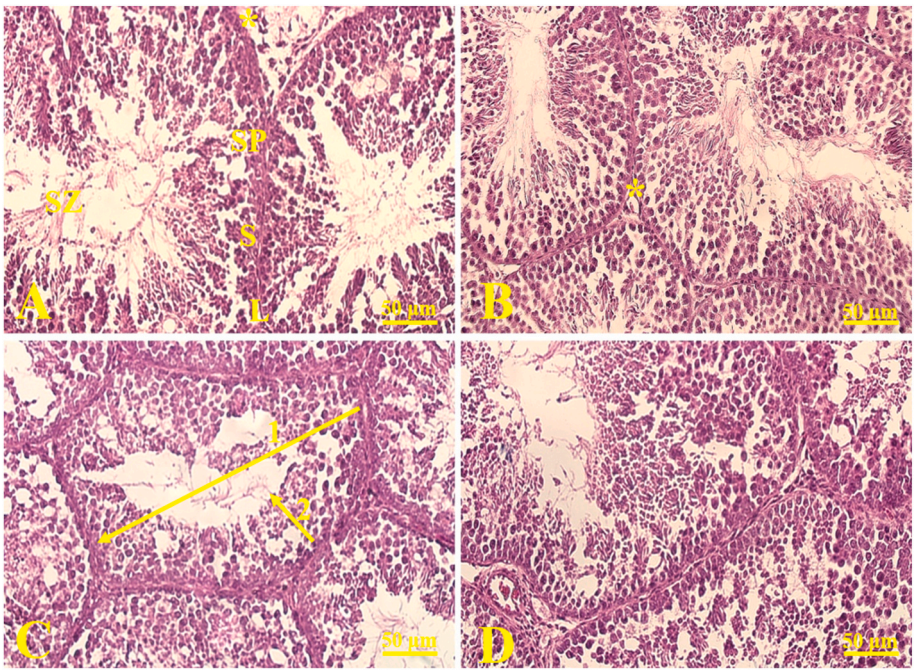

Fig. 1. Photomicrographs of cross-sections of testis of broiler breeder roosters fed barley sprouts powder (BS) and D-aspartic acid (DA) treatments. A, Control group; B, BS (2 % of basal diet); C, DA (200 mg/kg/BW); and D, BS + DA. [1]Seminiferous tubule diameter; [2]Seminiferous epithelium thickness; SP, Spermatogonia; L, Leydig cells; S, Sertoli cells; SZ, Sperm; *, Blood vessel. Hematoxylin and eosin stain, magnification of 200X.

Alterations in the testicular tissue of aging chickens affect reproduction [45]. Vacuoles in the testes of control roosters were an indicator of age-related increases in oxidative stress [1]. In addition, reduced seminiferous tubule diameter and epithelial thickness may have been due to an accumulation of reactive oxygen species in control roosters that can prevent the division and differentiation of spermatogonial cells. Oxidative damage includes modifications of cellular macromolecules, cell death by apoptosis or necrosis, plus structural damage [46]. Oxidative stress adversely affects seminiferous tubules and fertility [47].

It is well documented that polyphenols and flavonoids contain many hydroxyl groups (-OH) that exert antioxidant effects by binding to (scavenging) reactive oxygen species (ROS). We inferred that phenolic compounds in BS prevented oxidative damage in Sertoli and Leydig cells. Similarly, barley microgreens alleviated reproductive disorders, lipid profiles, histopathology, and oxidative stress in male rats [7]. Antioxidant compounds improve sperm concentration in seminiferous tubules by mitigating Sertoli cell damage [47]. In hens, phenolic compounds in barley sprouts increased fertility and the number of hatched chicks [6]. The increased number of reproductive cells in BS and DA treatments was attributed to increases in reproductive hormone concentrations, testicular blood flow, and Sertoli cell function. Due to their antioxidant properties, BS and DA increase the activity of enzymes such as catalase and peroxidase, which reduce free radicals [7]. Peroxidation of lipids, induction of vacuoles, and apoptosis of germ cells are

Table 7
The effects of experimental treatments on the mean serum concentrations of reproductive hormones, lipids, and MDA peroxidation index in aged roosters.

Treatments	T[a] (ng/mL)	LH[b] (miu/mL)	FSH[c] (iui/L)	Cholesterol	HDL[d]	LDL[e]	LDL/HDL	MDA[f] (nmol/mg)
BS (%)								
0	4.07	4.46^{b}	5.54	138.37^{a}	80.25^{b}	27.25^{a}	0.34^{a}	2.07^{a}
2	4.21	4.53^{a}	5.67	131.75^{b}	93.62^{a}	18.37^{b}	0.19^{b}	1.70^{b}
Pooled SEM	0.08	0.06	0.04	1.91	1.37	0.85	0.09	0.07
DA (mg/kg BW)								
0	3.98^{a}	4.46^{a}	5.55	133.37	86.37	22.87	0.27	1.92
200	4.30^{b}	4.53^{b}	5.65	136.75	87.50	22.75	0.26	1.85
Pooled SEM	0.08	0.06	0.04	1.91	1.37	0.85	0.09	0.07
BS × DA								
0 × 0	3.90^{b}	4.45^{b}	5.50^{b}	138.75^{a}	77.75^{b}	29.00^{a}	0.375^{a}	2.10^{a}
2 × 0	4.07^{ab}	4.46^{b}	5.76^{ab}	128.00^{b}	95.00^{a}	16.75^{b}	0.177^{c}	1.75^{bc}
0 × 200	4.25^{ab}	4.48^{b}	5.59^{ab}	138.00^{a}	82.75^{b}	25.50^{a}	0.307^{b}	2.05^{ab}
2 × 200	4.35^{a}	4.59^{a}	5.72^{a}	135.50^{ab}	92.25^{a}	20.00^{b}	0.215^{c}	1.65^{c}
Pooled SEM	0.11	0.09	0.05	2.71	1.93	1.20	0.01	0.10
P-value								
BS	0.252	0.001	0.050	0.031	0.001	0.001	0.001	0.003
DA	0.018	0.001	0.107	0.237	0.572	0.919	0.308	0.472
BS × DA	0.004	0.001	0.048	0.001	0.006	0.016	0.002	0.038

a-c: Within a column and cluster (between horizontal lines), means without a common superscript differed ($P < 0.05$).
[a] Testosterone.
[b] Luteinizing hormone.
[c] Follicle stimulating hormone.
[d] High density lipoprotein.
[e] Low density lipoprotein, and.
[f] Malondialdehyde. BS, barley sprout powder and DA, D-aspartic acid.

destructive outcomes of oxidative stress and free radical accumulation [2]. Therefore, thicker seminiferous epithelium and fewer vacuoles in roosters fed BS and DA were attributed to reductions in oxidative stress, including ROS formation. Long-term BS and DA can reduce oxidative stress, boost antioxidant enzymes (*SOD* and *GSH-P$_X$*), and improve fertility [6,40].

In testicular tissue of BS + DA treated roosters, MDA concentrations were decreased and there were significant increases in *GSH-P$_X$* and *SOD* activity; both are antioxidative enzymes [48] that presumably reduced oxidative stress and MDA concentrations in the testis, thereby improving sperm motility and morphology. Similarly, BS and DA increased *SOD* activities and reduced MDA concentrations in male rats [7] and roosters [18]. We inferred that BS may have promoted testicular growth by increasing antioxidant capacity and testosterone hormone secretion in adult roosters. Similarly, cinnamon (*Cinnamomum zeylanicum*) bark oil (natural antioxidant) suppressed heat stress (HS)-induced damage in sperm production, by enhancing testicular oxidant-antioxidant balance, testicular histopathologic structure, and ratios of testicular *BAX* and *BCL-2* in Japanese quail [49].

Apoptosis occurs during seasonal testicular regression and aging in most vertebrates, limiting the renewal of the germ cell population [50]. The ratio of *BAX* (apoptotic) and *BCL-2* (anti-apoptotic) proteins is a critical determinant of cell fate, with increased *BAX* accelerating cell death whereas increased *BCL-2* favors cell survival [51]. Apoptosis is also an indicator of DNA damage (including testicular germ cells), with free radicals increasing apoptosis in testicular germ cells [52], consistent with large numbers of apoptotic germ cells in Controls. However, mRNA expression of *BCL-2* was highest in the BS + DA group, which may account for the highest testis weight. Other studies also reported that BS up-regulated the expression of *BCL-2* [53].

Polyphenol bioactive compounds in BS may have multiple roles in cellular and plasma cholesterol metabolism, including stimulating adenosine monophosphate protein kinase (AMPK) activity and decreasing hydroxymethylglutaryl coenzyme A (HMG-COA) reductase enzyme activity in liver, thereby enhancing energy balance, homeostasis control, and lipid metabolism [54]. A high-cholesterol diet in rats caused Sertoli and Leydig cell malfunction, reducing sperm motility [55]. In addition, high plasma cholesterol concentrations adversely affect spermatogenesis and fertility, probably due to decreased testicular blood flow [55]. In this experiment, BS increased HDL and decreased cholesterol and LDL concentrations, presumably increasing blood flow to the testes and enhancing testicular function. In laboratory mice, HDL was positively correlated with Leydig cell diameter and spermatogenic epithelium thickness [56]. It seems that the Leydig cells may utilize HDL cholesterol for steroidogenesis. The most compelling arguments for using HDL as opposed to other plasma lipoproteins in testicular tissue are that compared to other lipoproteins, HDL has the highest proportion of phospholipids and the lowest proportion of triglycerides [57]. Hydrolysis of HDL produces copious quantities of phospholipids that readily traverse lipid barriers of most tissues, including the testis [58]. Sertoli cells are responsible for supplying reproductive cells in the testis with various nutrients, including lipids [59,60]. In addition, the barrier between seminiferous tubules and the testicular blood capillaries prevents the passage of LDL lipoproteins and only permits the exchange of HDL particles and cholesterol transfer to Sertoli cells [61]. Therefore, the cholesterol present in HDL, relative to other plasma lipid compounds, is better utilized to support developing reproductive cells in the testis.

Since this is the first report regarding the dietary inclusion of BS on the reproductive potential of these birds, further research is needed to clarify the mechanisms by which BS positively influences the reproductive potential of these birds.

5. Conclusions

In summary, dietary supplementation of BS + DA enhanced antioxidant status in the testes, with improved semen quality, reduced apoptosis, and improved histology. These beneficial effects were attributed to increased serum concentrations of LH and testosterone, plus improved lipid end points. Furthermore, BS + DA suppressed the expression of apoptosis-promoting genes. Oxidative stress induced by aging down-regulated mRNA expression of *SOD* or *GSH-Px*; however, antioxidant capability and reproductive potential were both improved when birds supplemented with BS + DA had up-regulation of relative expression of *GSH-Px* and *SOD* mRNA. Consequently, BS + DA has the potential to diminish or delay age-related subfertility in broiler breeder

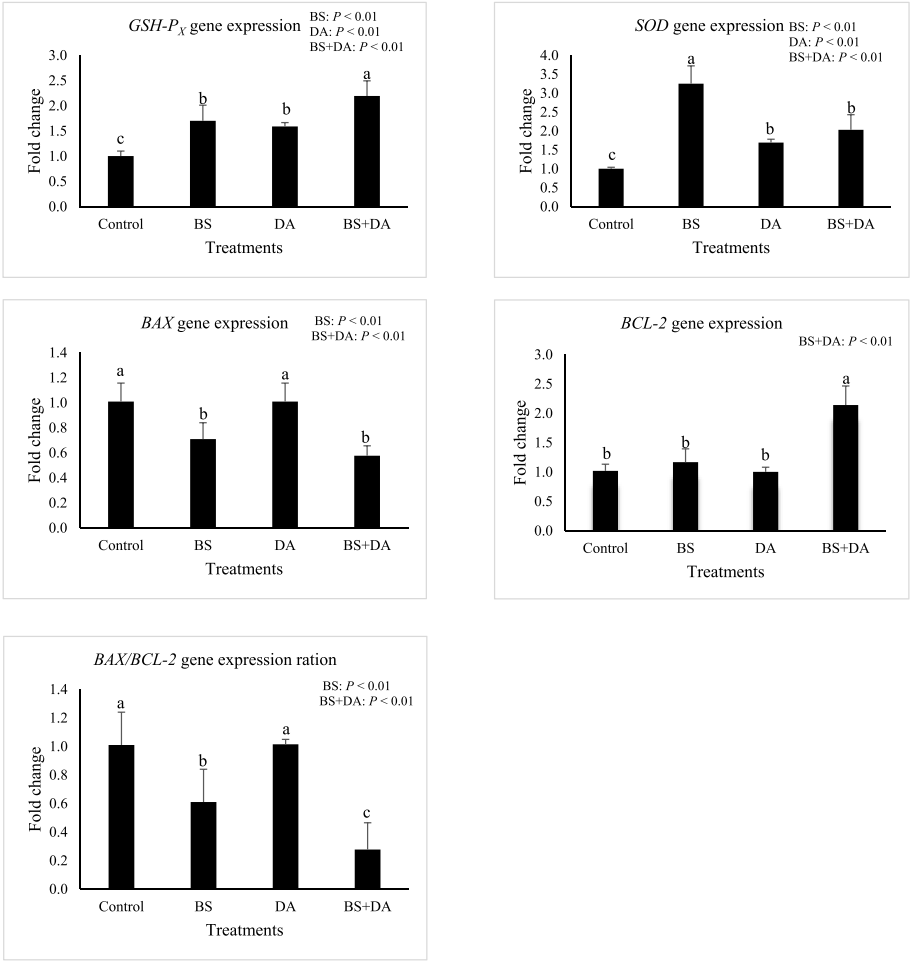

Fig. 2. Relative mRNA expression of *GSH-P$_X$*, Glutathione peroxidase; *SOD*, Superoxide dismutase; *BAX, BCL-2*-associated X protein; *BCL-2*, B-cell lymphoma-2, and *BAX/BCL-2* in testes of roosters with dietary supplementation of Barley Sprout powder (BS) and/or D-aspartic acid (DA) for 12 successive weeks: Control, BS (2 % of basal diet), DA (200 mg/kg/BW) and BS + DA. [a-c]Means without a common letter differed; $P < 0.05$ and $P < 0.01$ were significant and extremely significant differences, respectively. Significant *P*-values from 2 × 2 factorial analyses are listed; the model included the main effects of BS and DA and their interaction.

males.

CRediT authorship contribution statement

Sarallah Yarmohammadi Barbarestani: Investigation, Data curation, Writing – original draft. **Firooz Samadi:** Supervision, Project administration, Validation, Data curation, Writing – review & editing, Formal analysis. **Mojtaba Zaghari:** Conceptualization, Methodology. **Zarbakht Ansari Pirsaraie:** Methodology, Validation. **John P. Kastelic:** Writing – review & editing, Conceptualization, Validation.

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