Research Note: Dietary supplementation with pyrroloquinoline quinone disodium ($PQQ.Na_2$) improves oxidative status and semen quality in aging layer breeder roosters

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ABSTRACT As the antioxidant capacity of sperm declines with age in roosters, the objective of the present study was to determine the effects of different levels of pyrroloquinoline quinone disodium (**$PQQ.Na_2$**) on antioxidative and sperm quality parameters of aging layer breeder roosters. A total of ninety-six 63-wk-old Jinghong No. 1 layer breeder roosters were randomly assigned to 4 treatments (0, 0.5, 1, 2 mg/kg $PQQ.Na_2$) for 6 wk. Antioxidant activity and semen parameters were assessed biweekly. The dietary administration of $PQQ.Na_2$ significantly increased semen quality (semen volume, sperm motility, straightness, progressive motility, curvilinear velocity, straight-line velocity, and amplitude of lateral head displacement) and antioxidant capacity (T-SOD, GSH-Px, hydroxyl radical scavenging ability, and/or superoxide scavenging capacity) in seminal plasma in aging layer breeder roosters. Whereas, $PQQ.Na_2$ supplementations significantly decreased malondialdehyde (**MDA**) concentration in seminal plasma in aging layer breeder roosters. Supplementation with 1 mg/kg dietary $PQQ.Na_2$ as an antioxidant supplement could increase sperm quality and antioxidant activity of aging layer breeder roosters, while a higher dose (2 mg/kg) did not result in further increment.

Key words: aging breeding roosters, antioxidant status, sperm quality, pyrroloquinoline quinone

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INTRODUCTION

The rooster reproductive performance is vital for poultry production level, while sperm quality is an important factor affecting the rooster reproductive performance. Furthermore, for roosters, sperm quality declines strongly after 50 wk of age (Lagares et al., 2017). However, to maximize economic benefits, the poultry industry always expects roosters to maintain a high level of sperm quality for longer. Therefore, how to improve sperm quality in aging roosters becomes an urgent issue to be solved.

Sperm plasma is rich in polyunsaturated fatty acids (**PUFA**), which are sensitive to oxidative stress by reactive oxygen species (**ROS**) (Qi et al., 2019). Research shows that a high ROS level correlates with low sperm quality and male infertility (Akhlaghi et al., 2014). It is even worse in aging roosters. ROS accumulate in testes with age and continuously induces oxidative stress in cells. Therefore, oxidative stress is one of the vital factors in the declined reproductive performance of aged roosters. Though testis and sperm have weak antioxidant capacity, antioxidative compounds in testicular tissue and seminal plasma are capable of protecting sperm against ROS. Based on the above results, approaches have been used to improve semen quality and antioxidative capacity of testicular and seminal plasma, including the supplementation of dietary α-linolenic acid (Qi et al., 2019), turmeric by-product (Yan et al., 2017), and dried ginger rhizome (Akhlaghi et al., 2014). Nonetheless, more efficient replacements are needed to enhance reproductive performance in aging breeder roosters that particularly present age-related subfertility.

Pyrroloquinoline quinone (**PQQ**), which is an aromatic tricyclic o-quinone, was first found as a novel cofactor of bacterial dehydrogenases. $PQQ.Na_2$ has strong antioxidant properties and could protect organs and tissues against oxidative stress-induced lipid peroxidation. Furthermore, it is crucial for animals' growth and reproductive performance (Ikemoto et al., 2017). Previous reports show that $PQQ.Na_2$ improves

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reproductive performance in boar, and porcine models (Zhang et al., 2019; Zhu et al., 2019). Treatment with PQQ.Na_2 is a beneficial tool to maintain boar sperm linear motility via decreasing the mitochondrial ROS level (Zhu et al., 2019). However, little research has investigated the effects of dietary supplementation with PQQ.Na_2 on the sperm quality of aging roosters.

The current study aimed to investigate whether dietary PQQ·Na_2 supplementation could alleviate the oxidative status and sperm quality in aging roosters. We used 63-wk-old Jinghong No. 1 layer breeder roosters as an aging rooster model. Subsequently, the effects of dietary supplementation with PQQ·Na_2 on reproductive performance and oxidative stress parameters in the semen plasma were examined.

MATERIALS AND METHODS

Birds and Diets

All experimental protocols were approved by the Animal Care and Use Committee of Beijing University of Agriculture. Ninety-six 63-wk-old Jinghong No. 1 layer breeder roosters were randomly allocated to one of 4 dietary treatments. Each treatment had 6 replicates of 4 birds with 1 bird per cage. All birds were fed with a basal diet for 1 wk and then assigned to a corn-soybean meal-based diet containing 0, 0.5, 1, 2 mg/kg PQQ.Na_2 (for 6 wk. PQQ.Na_2 (purity, ≥99.5%) was provided by Shanghai Medical Life Sciences Research Center Co. Ltd (Shanghai, China). Diets were formulated to meet or exceed the nutritional requirements (Qi et al., 2019). All diets contained the same ingredients and were isonitrogenous (CP = 14.00 %) and isoenergetic (ME = 12.02 MJ·kg^{-1}). All birds had free access to water and diets during the test period.

Sample Collection

At 0, 2, 4, and 6 wk of the feeding trial, semen samples were collected from all roosters randomly selected from each replicate by dorso-abdonimal massage. Centrifuge the collected semen in a centrifuge at 4,000 rpm for 10 min. Slowly extract the upper semen and divide it into 1.5 mL centrifuge tubes, and store it in a refrigerator at −20°C for subsequent semen quality and antioxidant indices measurement.

Semen Quality Analysis

Semen volume was determined with a graduated collecting tube. Sperm concentration and sperm motility characteristics were analyzed by a computer-assisted sperm analyzer (**CASA**) system (WLJX-9000 Weili Color Sperm Analysis System, Weili New Century Science & Tech Dev., Beijing, China) and with settings adjusted to detecting avian spermatozoa (Qi et al., 2019). The specific analyzing procedures for semen samples were performed as described previously by Qi et al (Qi et al., 2019). The system provided data of the following 5 motility parameters: progressive motility (PMOT, %); curvilinear velocity (VCL, μm s^{-1}), which is the average velocity measured over the actual point-to-point track followed by the cell; straight-line velocity (VSL, μm s^{-1}), which represents the average velocity measured in a straight line from the beginning to the end of a track; straightness (STR, %) and amplitude of lateral head displacement (ALH, μm).

Sperm viability was evaluated using the eosin-nigrosin stain method (Akhlaghi et al., 2014). Sperm suspension smears were prepared by mixing 10 mL sperm sample with 20 mL of stain on a pre-warmed slide and spreading the stain with another slide. Viability was assessed by counting 200 cells using an Olympus BX51 microscope at a final magnification of 400 ×. Sperm with unstained heads of spermatozoa were considered viable while those with partial or complete purple-stained heads were considered to be dead.

Antioxidant Indices Analysis

The antioxidant capacities of semen were analyzed in duplicate. Total superoxide dismutase (**SOD**) activity was determined using the xanthine/xanthine oxidase system for superoxide anion generation. This anion reduces nitroblue tetrazolium to formazan, which was monitored at 550 nm. Malondialdehyde (**MDA**) was assayed using the thiobarbituric acid method by measuring spectrophotometrically reactive products at 532 nm. Glutathione peroxidase (**GSH-Px**) activity was determined based on the enzyme-catalyzed oxidation of GSH by cumene hydroperoxide coupled to the reduction of oxidized GSH by NADPH. Units of GSH-Px activity were expressed as micromole oxidized NADPH per minute. Hydroxyl radical scavenging ability and superoxide anion scavenging in seminal plasma capacity were measured according to the instructions in the commercial Colorimetric kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Statistics

All data were analyzed using SPSS 22.0 (IBM Corp., NY). Data related to the effect of dietary PQQ.Na_2 levels on semen quality and antioxidant capacity were analyzed by one-way ANOVA analysis. 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

PQQ, which is characterized as a vitamin-like redox cofactor, is an effective antioxidant, protecting organs and tissues against oxidative stress-induced lipid peroxidation (Zhang et al., 2019a), and it is crucial for animal's growth and reproductive performance (Ikemoto et al.,

2017). Previous reports showed that PQQ.Na_2 improves reproductive performance in boar and porcine models (Zhang et al., 2019; Zhu et al., 2019). Furthermore, research showed that reproductive performance (e.g., fertility rate) is positively correlated with sperm quality (Tsakmakidis et al., 2010). Therefore, this study was designed to investigate the effect of dietary PQQ.Na_2 on semen quality and antioxidant activity. Our results showed that dietary PQQ.Na_2 successfully improved semen volume, sperm motility, straightness, progressive motility, curvilinear velocity, straight-line velocity, and amplitude of lateral head displacement (Table 1). With increasing dietary levels of PQQ.Na_2, semen volume was significantly increased linearly in dietary PQQ.Na_2 supplementation from 0 to 2 mg/kg ($P < 0.01$), and at 2, 4, and 6 wk, semen volume increased by 37.5, 91.7, and 235%, respectively. At 4 and 6 wk, sperm motility significantly increased with PQQ.Na_2 supplementation from 0 to 1 mg/kg, while there was no further increase in PQQ.Na_2 supplementation from 1 to 2 mg/kg. Semen concentration was not affected by supplemental PQQ.Na_2 ($P > 0.05$) at 2, 4, and 6 wk, and straightness only significantly increased after dietary PQQ.Na_2 supplementation at 6 wk. Progressive motility, curvilinear velocity, straight-line velocity, and amplitude of lateral head displacement were significantly higher in the 2 mg/kg PQQ.Na_2 group than that in the control group (0 mg/kg PQQ.Na_2 group) at 2, 4, and 6 wk (expecting for progressive motility). This was in line with a previous study (Yan et al., 2017). Direct evidence shows that antioxidant PQQ.Na_2 supplementation could largely rescue reproductive aging phenotype (He et al., 2021). This might be because spermatozoa are particularly rich in PUFA, which predisposes them to lipid peroxidation by ROS, and this is correlated with male infertility. Therefore, the antioxidant ability was analyzed in seminal plasma to clarify the effect of dietary PQQ.Na_2 supplementation on the aging layer of breeder sperm in the following study.

In the current study, T-SOD, GSH-Px, and hydroxyl radical scavenging ability significantly increased after 4-wk PQQ.Na_2 supplementation. In seminal plasma (Table 2), compared to the control group, T-SOD, GSH-Px, and hydroxyl radical scavenging ability were significantly higher in 0.5 and 1 mg/kg PQQ.Na_2 group at 4 and 6 wk, while they were not significant further increment in 2 mg/kg PQQ.Na_2 group (expecting hydroxyl radical scavenging ability in 6 wk). MDA significantly decreased in all PQQ.Na_2 groups, compared to the control group. This was in line with the results of their semen quality that sperm motility considerably enhanced after 4-wk PQQ.Na_2 supplementation. Zhu et al also demonstrated that PQQ.Na_2 treatment maintained sperm linear motility (Zhu et al., 2019). The sperm membrane is rich in PUFA, which is easy to suffer lipid peroxidation by ROS, leading to a decline in sperm motility (Yan et al., 2017). Furthermore, research shows that avian sperm has greater concentrations of PUFA than other species (Surai et al., 1998), as a result, the reductions of sperm motility resulting from ROS activity

Table 1. Effect of pyrroloquinoline quinone disodium (PQQ.Na_2) supplementation on semen quality in layer breeder roosters.

Items	Time/wk	Supplemental levels / (mg/kg)					*P*-value	
		0	0.5	1	2	SEM	Linear	Quadratic
Semen volume (mL/rooster)	0	0.25	0.26	0.26	0.27	0.00	0.04	0.93
	2	0.24^{c}	0.28^{b}	0.30^{ab}	0.33^{a}	0.01	<0.01	0.61
	4	0.24^{c}	0.26^{c}	0.33^{b}	0.46^{a}	0.18	<0.01	<0.01
	6	0.20^{d}	0.29^{c}	0.44^{b}	0.67^{a}	0.04	<0.01	<0.01
Sperm motility (%)	0	81.52	82.91	83.22	82.63	1.27	0.77	0.72
	2	82.33	82.52	85.91	83.9	1.00	0.39	0.6
	4	83.41^{c}	90.71^{b}	95.25^{a}	95.64^{a}	1.14	<0.01	<0.01
	6	80.73^{c}	89.51^{b}	92.22^{ab}	96.58^{a}	1.35	<0.01	0.11
Sperm concentration ($\times 10^8$ spermatozoa/mL)	0	10.64	10.77	10.81	10.82	0.15	0.68	0.85
	2	11.28	11.41	11.56	11.57	0.11	0.35	0.82
	4	11.53	11.54	11.99	12.08	0.13	0.07	0.86
	6	12.45	12.49	12.50	12.91	0.19	0.45	0.65
Straightness (%)	0	79.44	80.60	80.38	80.49	1.08	0.78	0.82
	2	82.18	83.92	84.13	86.51	0.78	0.07	0.84
	4	86.73	86.00	87.32	89.08	0.73	0.22	0.41
	6	88.93^{c}	99.43^{b}	121.14^{a}	123.13^{a}	3.07	<0.01	<0.01
Progressive motility (%)	0	47.45	47.44	48.17	48.41	0.62	0.55	0.92
	2	48.19^{c}	50.40^{bc}	52.63^{ab}	55.85^{a}	0.73	<0.01	0.59
	4	54.07^{b}	51.57^{b}	50.93^{b}	59.00^{a}	0.88	0.02	<0.01
	6	55.13	56.15	58.45	57.24	0.62	0.12	0.37
curvilinear velocity (μm/s)	0	69.39	70.54	71.41	71.67	0.68	0.23	0.76
	2	69.60^{b}	70.68^{b}	72.98^{ab}	74.83^{a}	0.72	<0.01	0.76
	4	69.62^{b}	72.97^{ab}	73.25^{ab}	75.16^{a}	0.72	<0.01	0.57
	6	70.21^{b}	73.45^{ab}	74.46^{ab}	76.32^{a}	0.93	0.03	0.71
straight-line velocity (μm/s)	0	39.96	40.04	40.48	41.94	0.54	0.21	0.54
	2	41.62^{b}	43.22^{ab}	43.27^{ab}	45.23^{a}	0.50	0.01	0.84
	4	41.49^{b}	43.67^{b}	44.00^{ab}	47.44^{a}	0.62	<0.01	0.5
	6	42.22^{b}	44.13^{b}	44.45^{ab}	48.46^{a}	0.68	<0.01	0.32
Amplitude of lateral head displacement (μm)	0	2.15	2.22	2.20	2.19	0.07	<0.01	<0.01
	2	2.35^{b}	2.48^{b}	2.52^{b}	2.69^{a}	0.03	<0.01	0.54
	4	2.48^{c}	2.53^{bc}	2.75^{b}	3.00^{a}	0.05	0.083	<0.01
	6	2.61^{c}	2.61^{c}	3.01^{b}	3.15^{a}	0.05	<0.01	0.04

^{abc}Means within a row with no common superscripts differ significantly ($P < 0.05$).

Table 2. Effects of pyrroloquinoline quinone disodium ($PQQ.Na_2$) supplementation on antioxidant activity in seminal plasma in layer breeder roosters.

Index	Time/wk	PQQ-level (mg/kg)				SEM	*P*-value	
		0	0.5	1	2		Linear	Quadratic
T-SOD (U/mL)	0	112.22	112.56	113.04	112.58	1.51	0.9	0.92
	2	109.10	110.44	111.05	111.99	1.59	0.54	0.95
	4	110.83^{c}	135.39^{b}	138.91^{ab}	149.70^{a}	3.27	<0.01	0.03
	6	130.46^{b}	135.69^{b}	148.77^{a}	151.39^{a}	2.31	<0.01	0.67
GSH-Px (U/mL)	0	480.47	481.81	483.15	482.68	2.28	0.72	0.85
	2	492.68	499.14	498.69	505.62	2.25	0.06	0.96
	4	605.87^{c}	655.41^{b}	667.58^{ab}	690.76^{a}	7.45	<0.01	0.11
	6	732.01^{c}	780.49^{b}	799.60^{ab}	850.35^{a}	10.07	<0.01	0.91
Hydroxyl radical scavenging ability (U/mL)	0	450.87	445.26	448.36	449.14	4.45	0.96	0.74
	2	485.48	490.45	488.47	489.15	4.11	0.82	0.81
	4	653.27^{c}	695.45^{bc}	699.44^{b}	750.13^{a}	8.81	<0.01	0.7
	6	688.94^{b}	701.56^{b}	725.15^{ab}	755.11^{a}	7.04	<0.01	0.4
Superoxide anion scavenging capacity (U/mL)	0	285.06	288.44	289.08	290.42	2.61	0.51	0.86
	2	304.09	305.11	303.95	305.45	17.64	0.99	1.00
	4	334.36	330.55	335.41	336.48	2.82	0.68	0.69
	6	350.46^{b}	360.10^{ab}	360.51^{ab}	380.44^{a}	3.62	<0.01	0.41
MDA (nmol/mL)	0	5.12^{b}	5.90^{a}	5.35^{b}	5.85^{a}	0.08	0.01	0.16
	2	4.45^{a}	3.66^{b}	3.01^{c}	3.70^{b}	0.12	<0.01	<0.01
	4	4.54^{a}	3.45^{b}	3.50^{b}	3.44^{b}	0.11	<0.01	<0.01
	6	3.09^{a}	3.11^{a}	2.74^{b}	2.32^{c}	0.08	<0.01	0.04

^{abc}Means within a row with no common superscripts differ significantly ($P < 0.05$).

are also greater than those in other species. Plus, $PQQ.Na_2$, which is a mitochondria-targeting antioxidant, might improve sperm quality via decreasing mitochondrial ROS level (Zhu et al., 2019). Therefore, dietary $PQQ.Na_2$ might improve sperm quality in aging roosters directly through enhancing semen antioxidant status.

The possible mechanism of $PQQ.Na_2$ improving semen quality in aging roosters could result from its strong antioxidant properties, as discussed above. This study might be the first time that has investigated the effect of supplementation with $PQQ.Na_2$ on aging breeder roosters and reported its most effective level. Based on the above results, dietary $PQQ.Na_2$ supplementation with 1 mg/kg significantly improved semen and sperm parameters in aging breeder roosters, and higher doses (2 mg/kg) did not lead to further improvement, although some antioxidant parameters continuously increased until dietary $PQQ.Na_2$ was supplemented with 2 mg/kg for most of them. This indicated that the improvement in antioxidant ability could only enhance sperm quality of aging roosters to some extent, and could not increase continuously with the improvement of antioxidant status.

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DISCLOSURES

The authors declare that they have no competing interests.

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