



Biochemical, Molecular, and Genetic Mechanisms in Nutrition

Optimum Doses and Forms of Selenium Maintaining Reproductive Health via Regulating Homeostasis of Gut Microbiota and Testicular Redox, Inflammation, Cell Proliferation, and Apoptosis in Roosters[☆]



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ABSTRACT

Background: There is a U-shaped relationship between dietary selenium (Se) ingestion and optimal sperm quality.

Objectives: This study aimed to investigate the optimal dietary dose and forms of Se for sperm quality of breeder roosters and the relevant mechanisms.

Methods: In experiment 1, 18-wk-old Jingbai laying breeder roosters were fed a Se-deficient base diet (BD, 0.06 mg Se/kg), or the BD + 0.1, 0.2, 0.3, 0.4, 0.5, or 1.0 mg Se/kg for 9 wk. In experiment 2, the roosters were fed the BD or the BD + sodium selenite (SeNa), seleno-yeast (SeY), or Se-nanoparticles (SeNPs) at 0.2 mg Se/kg for 9 wk.

Results: In experiment 1, added dietary 0.2 and 0.3 mg Se/kg led to higher sperm motility and lower sperm mortality than the other groups at weeks 5, 7, and/or 9. Furthermore, added dietary 0.2–0.4 mg Se/kg produced better testicular histology and/or lower testicular 8-hydroxy-deoxyguanosine than the other groups. Moreover, integrated testicular transcriptomic and cecal microbiomic analysis revealed that inflammation, cell proliferation, and apoptosis-related genes and bacteria were dysregulated by Se deficiency or excess. In experiment 2, compared with SeNa, SeNPs slightly increased sperm motility throughout the experiment, whereas SeNPs slightly reduced sperm mortality compared with SeY at week 9. Both SeY and SeNPs decreased malondialdehyde in the serum than those of SeNa, and SeNPs led to higher glutathione peroxidase (GPX) and thioredoxin reductase activities and GPX1 and B-cell lymphoma 2 protein concentrations in the testis compared with SeY and SeNa.

Conclusions: The optimal dietary Se dose for reproductive health of breeder roosters is 0.25–0.35 mg Se/kg, and SeNPs displayed better effects on reproductive health than SeNa and SeY in laying breeder roosters. The optimal doses and forms of Se maintain reproductive health of roosters associated with regulation intestinal microbiota homeostasis and/or testicular redox balance, inflammation, cell proliferation, and apoptosis.

Keywords: selenium, reproductive health, transcriptome, microbiome, roosters

Abbreviations: 8-OHdG, 8-hydroxy-deoxyguanosine; BCL-2, B-cell lymphoma 2; BD, base diet; DEG, differentially expressed gene; DIO, iodothyronine deiodinase; ERK, extracellular signal-regulated kinase; FSH, follicle-stimulating hormone; GPX, glutathione peroxidase; JNK, c-Jun N-terminal kinase; KEGG, Kyoto Encyclopedia of Genes and Genomes; LEfSe, linear discriminant analysis effect size; LH, luteinizing hormone; MDA, malondialdehyde; MSRB1, methionine sulfoxide reductase B1; SELENO, selenoprotein; TXNRD, thioredoxin reductase.

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Introduction

Fertility is a major factor affecting the economic outcome from farm animals [1]. Generally, the quality of the inseminated semen modulates the fertility of poultry flocks and is influenced by several variables, including breed, age, environment, and nutrition [2]. Numerous studies have shown that sperm quality is affected by oxidative stress induced by environmental and metabolic stress [3,4]. High amounts of PUFAs in avian spermatozoa membranes increase their susceptibility to oxidative stress, which influences the quality and fertility potential of sperm [5]. Therefore, application of nutrients with antioxidant function to control redox balance and improve sperm quality and fertility in roosters has drawn much attention [6,7].

Selenium (Se) is an essential micronutrient for life, with demonstrated pivotal roles in antioxidant defense and redox control [8]. The biological functions of Se in poultry have mainly been attributed to the presence of the 21st amino acid, seleno-cysteine, in 25 selenoproteins [9]. Thus, Se has often been used as an antioxidant in feed for animal health, but suboptimal Se consumption is a hidden health issue. In addition, the gut microbiota plays vital roles in spermatogenesis and has been shown to affect the host's Se utilization by competing with the host for Se when its availability becomes limiting [10]. Moreover, there is a U-shaped relationship between Se status and optimal health [11,12], especially in male reproduction [13]. Previous studies have demonstrated that Se makes up the bulk of mitochondria located in the sperm midpiece. Mitochondria are extremely susceptible to oxidative stress, and Se deficiency leads to abnormalities in mitochondrial structure and function, which induces decreased sperm motility, abnormal morphology, and infertility [14,15]. The testis accumulates the highest Se concentration per dry mass of all tissues when dietary Se is sufficient, and it has priority for Se accumulation when dietary Se is insufficient [16,17]. Previous studies have identified a strong relationship between Se status and sperm quality and revealed that either Se deficiency (<0.05 mg Se/kg) or excess (>1.0 mg Se/kg) impairs testis development and reduces sperm quality [18–21]. However, the optimal dose of dietary Se for roosters has not been well established. Moreover, the molecular mechanism by which Se deficiency or excess alters sperm quality has not been fully elucidated.

The form of Se in the diet is also of great importance for animal health. Usually, it is standard practice to supplement feeds with inorganic Se, such as sodium selenite (SeNa), or organic Se, such as seleno-yeast (SeY), to avoid Se deficiency and fulfill the dietary requirements of poultry [22]. Advances in nanotechnology have provided several new effective forms of dietary supplementation with low toxicity and high bioavailability [23,24]. Among them, Se-nanoparticles (SeNPs) exhibit superior antioxidant and anti-inflammatory activities compared with selenates, selenites, and SeY due to their large surface area, effective surface activity, high catalytic efficiency, strong absorption, and low toxicity [25]. Therefore, determining whether SeNPs exert unique effects on the reproductive health of roosters compared with SeNa and SeY is important.

The Jingbai laying breeder strain has good performance and egg quality but relatively low-sperm quality. Thus, we selected Jingbai laying breeder roosters to determine the optimal dietary

Se concentration and Se sources that improve sperm quality and investigate the relevant role of Se in spermatogenesis.

Methods

Experimental design

The animal study protocol was approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University. In experiment 1, a total of 210 18-wk-old Jingbai laying breeder roosters were randomly allocated into 7 treatment groups with 10 replicates of 3 birds each. The 7 groups of roosters were fed a base diet (BD, 0.06 mg Se/kg; [Supplemental Table 1](#)) or the BD supplemented with 0.1, 0.2, 0.3, 0.4, 0.5, or 1.0 mg Se/kg as Na_2SeO_3 . The analyzed Se concentrations in the BD, 0.1Se, 0.2Se, 0.3Se, 0.4Se, 0.5Se and 1.0Se were 0.06, 0.15, 0.24, 0.34, 0.45, 0.57, and 1.09 mg/kg, respectively. The BD was prepared with nutrients following the recommendations of the National Research Council [26]. The BD was composed of corn and soybean grown in Hanyuan, Sichuan, China, a Se-deficient area, and was not supplemented with additional Se. The breeder roosters were feed-restricted to 70–85 g feed/day from 18 to 27 wk of age and had free access to water. The health status and mortality of the roosters were monitored daily during the trial. At the end of week 7, 6 roosters from each group were randomly selected and killed for blood and testis sample collection. The testis samples were washed with ice-cold isotonic saline and then snap-frozen in liquid nitrogen until use.

In experiment 2, a total of 120 18-wk-old Jingbai laying breeder roosters were randomly allocated into 4 treatment groups with 10 replicates of 3 birds each. Experiment 2 used the same BD as experiment 1. The birds were fed the BD or the BD supplemented with 0.2 mg Se/kg as SeNa (Retorte GmbH), SeY (Alkosel and Lallemand), or SeNPs (Beijing Deyuanshun Biotechnology Co., Ltd.) for 9 wk. The analyzed Se concentrations in BD, SeNa, SeY, and SeNPs were 0.06, 0.24, 0.25, and 0.27 mg/kg, respectively. At the end of week 5, 5 roosters from each group were randomly selected and killed for blood and testis sample collection. The animal feeding program and sample collection procedure were the same as described for experiment 1.

Sperm quality assay

Semen samples were collected weekly from roosters (10 birds/group) using a massage technique [27]. Sperm motility, mortality, and density were evaluated with a Mylan Automatic Sperm Quality Analysis System (ML-100BJZ; Nanning Songjing Tianlun Biotechnology Co., Ltd.).

Se concentration, sex hormones, testicular redox status, and histologic analysis

The feed, serum, and testis Se concentrations were measured, as described previously [28]. Briefly, after the samples were digested with a nitric acid–perchloric acid mixture, a hydride generation atomic fluorescence spectrometer (AF-610B; Beijing Rayleigh Analytical Instrument Corporation) was used to determine the Se concentration. Serum testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) concentrations were measured with ELISA kits (LE-CH365, LE-CH390, and LE-CH349; Lai Er Bio-Tech). Testicular malondialdehyde (MDA) concentrations and glutathione

peroxidase (GPX) activity were determined with specific assay kits (A003-1-2 and A005-1-2; Nanjing Jiancheng Bioengineering Institute). Thioredoxin reductase (TXNRD) activity was determined with a specific assay kit (TRXR-1-W; Suzhou Comin Biotechnology Co., Ltd.). The 8-hydroxy-deoxyguanosine (8-OHdG) concentration was determined with an ELISA kit (MM-3325601; Jiangsu Meimian Industrial Co., Ltd.). The testicular samples were examined microscopically after being fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin and eosin [20].

Transcriptome analysis

RNA-seq analysis of testis samples from the BD, 0.2 mg Se/kg, and 1.0 mg Se/kg groups in experiment 1 was performed, as described previously [29]. RNA was extracted from 4 samples from each group and then submitted to Wuhan Seqhealth Technology Co., Ltd. After RNA quality assessment (RNA integrity number >7.5), sequencing was performed on an Illumina HiSeq platform, and 150-bp paired-end reads were generated. Reads of each sample were aligned to the chicken genome (GRCg6a/galGal6) using HISAT2 (v2.1.0). Feature Counts (v1.6) was used to count the numbers of reads mapped to each gene. Differentially expressed genes (DEGs) were identified with the DESeq2 R package (1.32.0) using a *P* value of < 0.05 and a fold-change of >1.5 or <0.7. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEGs were implemented with the Cluster Profiler (4.0.2) R package. The Benjamini Hochberg false discovery rate was used for multiple testing corrections (false discovery rate < 0.05).

Microbiomic analysis

Cecal digesta samples from the BD, 0.2 mg Se/kg, and 1.0 mg Se/kg groups from experiment 1 were used for microbiomic analysis. Microbial DNA was extracted according to the protocol described previously [30]. Briefly, 6 replicates were randomly selected from the 3 groups. Each sample was amplified with the V3-V4 primers. The pooled library was sequenced on the Illumina HiSeq platform (PE250). Raw fastq files were quality-filtered and taxonomically analyzed using QIIME2 (v2019.7). Linear discriminant analysis coupled with linear discriminant analysis effect size (LEfSe) was performed to identify which bacterial taxa were different among the specific groups with Linear discriminant analysis scores > 2.

qRT-PCR and western blotting analysis

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. qRT-PCR was employed to evaluate the relative changes in selenoprotein (*SELENO*) gene expression, as described previously [31]. The primers for the assayed genes and the reference gene β -actin are presented in Supplemental Table 2. Relative mRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method, and the relative abundance was normalized to the BD group (set as 1). Total proteins were extracted from the testis using radioimmunoprecipitation assay buffer with 1.0% phenylmethylsulphonyl fluoride (Beyotime Biotechnology), and western blotting analysis of the pertinent samples was conducted, as described previously [32]. The primary antibodies used for the analyses are presented in

Supplemental Table 3. All protein levels were normalized to the housekeeping protein β -actin. Densitometric quantification of the western blotting bands was performed using Image J software (National Institutes of Health).

Integrated transcriptome and microbiome analysis

Spearman correlation was used for correlation analysis with normalized gene expression data and microbiome relative abundance [33]. DEGs from the BD and 1.0 mg Se/kg groups compared with the 0.2 mg Se/kg group and top 100 microbiome abundance at the genus level were subjected to correlation analysis. Significant correlations (absolute correlation > 0.6 and *P* value < 0.05) were visualized as a heatmap/network using R v4.1.

Statistical analysis

Except for the transcriptome and microbiomic analyses indicated above, all data were analyzed with analysis of variance followed by least significant difference post hoc test for multiple comparisons. The data are presented as means $\pm$ SE, and *P* value of <0.05 was considered to indicate statistical significance. Statistical analysis and image presentation were performed with GraphPad Prism version 9.0 (GraphPad Software).

Results

The effects of the dietary Se dose on body weight, sperm quality, sex hormones, and histopathology

There were no effects (*P* $\geq$ 0.05) of the diets on the initial and final body weights and the testis index of breeder roosters (Figure 1A–C). Compared with the BD, dietary supplementation with 0.1–0.4 mg Se/kg led to higher (*P* < 0.05) sperm motility (10.6%–38.1%) and lower (*P* < 0.05) sperm mortality (60.5%–96.5%). Throughout the experiment, dietary supplementation with 0.5 mg Se/kg provided better (*P* < 0.05) sperm motility (7.3%–9.2%) and lower sperm mortality (47.6%–75.1%) at weeks 7 and 9, and dietary supplementation with 1.0 mg Se/kg reduced (*P* < 0.05) sperm mortality (24.6%–31.9%) at weeks 7 and 9 (Figure 1D, E). Notably, dietary supplementation with 0.2 mg Se/kg produced the best sperm motility and mortality outcomes at some of the experimental time points (Figure 1D, E). Strikingly, the BD and 1.0 mg Se/kg groups had higher sperm density than some of the other groups at weeks 5 and/or 9 (Figure 1F).

Compared with the BD, dietary supplementation with $\geq$ 0.3 mg Se/kg reduced (*P* < 0.05) serum testosterone by 31.4% to 45.1% (Figure 1G). Compared with the BD, dietary supplementation with $\geq$ 0.2 mg Se/kg decreased (*P* < 0.05) the serum FSH concentration by 22.8% to 31.4% (Figure 1H). However, the serum LH concentration was not affected by dietary Se (Figure 1I). There were histopathologic changes in the testis of breeder roosters fed the BD, including detachment of the spermatogenic epithelium from the basal membrane; disordered arrangement of spermatogenic epithelial cells; a reduction in the luminal cell density; and an increase in interstitial and spherical sperm cells, degenerated sperm, and remnants (Figure 1J). Meanwhile, there was an increase in spherical sperm cells, degenerated sperm, and remnants in the testis of breeder

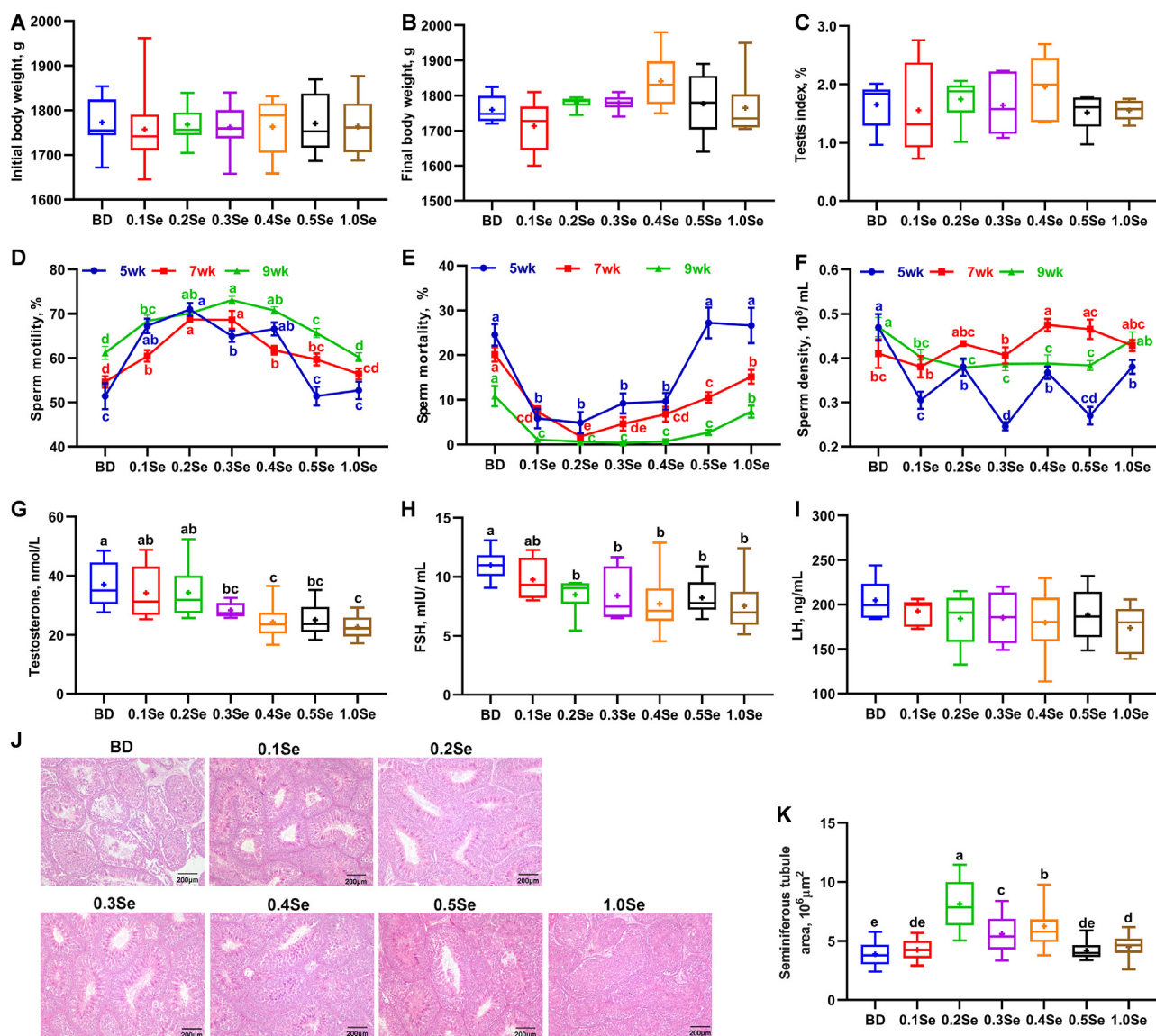


FIGURE 1. Effects of dietary Se dose on (A–C) growth performance, (D–F) sperm quality, (G–I) serum hormone concentrations, and (J–K) testicular histopathology of breeder roosters. Data are presented as boxplots ranging from minimum to maximum or mean $\pm$ SE ($n = 10$). Labeled means without a common letter differ significantly ($P < 0.05$). Abbreviations: BD, base diet; Se, selenium; 0.1Se, 0.2Se, 0.3Se, 0.4Se, 0.5Se, and 1.0Se, BD supplemented with 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 mg Se/kg, respectively.

roosters in the 1.0 mg Se/kg group (Figure 1J). Moreover, dietary supplementation with 0.2–0.4 mg Se/kg produced a larger area ($P < 0.05$) of spermatogenic tubules than the other groups (Figure 1K).

The effects of the dietary Se dose on the Se concentration, redox status, and selenogenome expression in the testis

Compared with the BD, the plasma and testis Se concentrations increased gradually ($P < 0.05$) by 16.7% to 180.7% and 18.4% to 134.7%, respectively, as the dietary Se concentration increased (Figure 2A, B). Compared with the BD, dietary supplementation with 0.3 and 0.4 mg Se/kg decreased ($P < 0.05$) the 8-OHdG concentration in the testis (25.5%–29.8%), whereas dietary supplementation with 1.0 mg Se/kg increased ($P < 0.05$)

the 8-OHdG concentration in the testis compared with all other groups (Figure 2D). Meanwhile, dietary supplementation with 0.1 to 0.5 mg Se/kg increased ($P < 0.05$) the testicular TXNRD activity (24.3%–38.4%) compared with the BD group (Figure 2F). However, the testicular MDA concentration and GPX activity were not affected ($P \geq 0.05$) by the dietary Se dose (Figure 2C, E).

Among the genes in the testis selenogenome, 19 were affected by dietary Se supplementation (Figure 2G; Supplemental Table 4). Compared with the BD, dietary supplementation with 0.1 mg Se/kg did not affect ($P \geq 0.05$) the expression of any selenoprotein gene. Dietary supplementation with 0.2–0.4 mg Se/kg upregulated ($P < 0.05$), *SELENOK*, *SELENOP*, *GPX3*, *SELENOH*, *SELENOU*, *MSRB1*, *SELENOO*, *SELENOP2*, and/or *TXNRD3*. The latter 4 genes plus *DIO1*, *SELENOM*, and *TXNRD2* were upregulated ($P < 0.05$), but *SELENOW* was downregulated

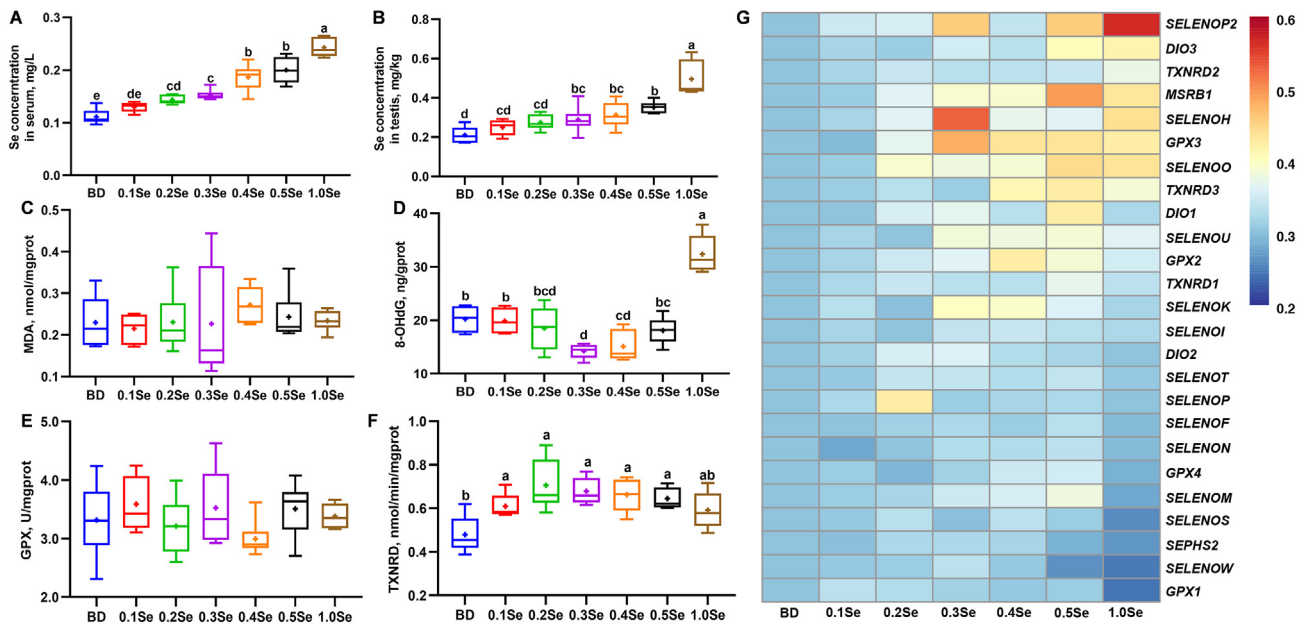


FIGURE 2. Effects of dietary Se dose on (A–B) Se concentration, (C–F) testicular redox index, and (G) testicular selenoprotein gene expression of breeder roosters. Data are presented as boxplots ranging from minimum to maximum ($n = 6$). mRNA expression in the BD group was set as 1 for fold-change calculation. Data are presented as a heatmap showing $\log_{10}$ (fold-change + 1) of gene expression. Labeled means without a common letter differ significantly ($P < 0.05$). Abbreviations: 8-OHdG, 8-hydroxy-deoxyguanosine; BD, base diet; GPX, glutathione peroxidase; MDA, malondialdehyde; MSRB1, methionine sulfoxide reductase B1; Se, selenium; 0.1Se, 0.2Se, 0.3Se, 0.4Se, 0.5Se, and 1.0Se, BD supplemented with 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 mg Se/kg, respectively; SELENO; selenoprotein; SEPHS2, selenophosphate synthetase 2; TXNRD, thio-reductase.

($P < 0.05$) by dietary supplementation with 0.5 and/or 1.0 mg Se/kg. However, *DIO2*, *GPX1*, *GPX2*, *SELENOF*, *SELENOI*, *SELENON*, *SELENOT*, and *SEPHS2* gene expression was not affected ($P \geq 0.05$) by the dietary Se dose.

Transcriptomic and microbiomic insights regarding how dietary Se deficiency and excess affect sperm quality

To better understand the molecular biology of how Se affects the reproductive system of breeder roosters, the testicular transcriptome and cecal microbiome were further analyzed in the BD, 0.2Se, and 1.0Se groups (Figure 3). Compared with the 0.2Se group, BD upregulated 215 genes and downregulated 152 genes (Figure 3A, Supplemental File 1), whereas 1.0Se upregulated 149 genes and downregulated 197 genes in the testis of breeder roosters (Figure 3B, Supplemental File 2). KEGG pathway analysis assigned the above DEGs in BD and 1.0Se, compared to 0.2Se, respectively; the results were shown in Supplemental Files 3 and 4. Notably, 56 DEGs were affected by the 2 extreme Se doses (Figure 3C, Supplemental File 5) and were assigned to 12 pathways (Figure 3D, Supplemental File 6). Among them, the neuroactive ligand–receptor interaction, cell adhesion molecules, the calcium signaling pathway, pyrimidine metabolism, and the mitogen-activated protein kinase (MAPK) signaling pathway were the most abundant pathways.

The potential involvement of MAPK/c-Jun-N-terminal kinase (JNK)/extracellular signal-regulated kinase (ERK) signaling in the Se-mediated effects on the breeder rooster reproductive system were analyzed by western blotting (Figure 3D). Specifically, compared with the BD, 0.5Se, and 1.0Se groups, dietary

supplementation with 0.1 to 0.4 mg Se/kg decreased ($P < 0.05$) phospho-JNK (p-JNK) in the testis (Figure 3E). Interesting, there was more phospho-ERK (p-ERK) in the testis of the 0.2Se, 0.3Se, and 0.4Se groups ($P < 0.05$) than those of BD, 0.1Se, and 1.0Se groups. Compared with the BD, dietary supplementation with 0.2–0.5 mg Se/kg increased ($P < 0.05$) B-cell lymphoma 2 (BCL-2) protein production in the testis (Figure 3E). However, the protein production of JNK, ERK, cleaved-caspase 3, and caspase3 were not affected ($P \geq 0.05$) by the dietary Se dose (Figure 3E).

There was no difference in α diversity and β -diversity (Supplemental Figure 1). The effects of Se on the gut microbiota composition are shown in Figure 3F and G. LEfSe analysis was used to identify which bacterial taxa were distinctly different among the 3 groups. The results showed that BD reduced the relative abundance of *Firmicutes* at the phylum level and increased the relative abundance of *Eggerthellaceae* at the family level compared to the 0.2Se group (Figure 3F, Supplemental File 7). Meanwhile, the 1.0Se group reduced the relative abundance of *Campylobacterota* at the phylum level and *Terrisporobacter*, *Campylobacteraceae*, *Candidatus_Arthromitus*, *Clostridium_sensu_stricto_1*, and *Candidatus_Soleaferrea* at the genus level and increased the relative abundances of *Ruminococcus_torques_group* and *Merdibacter* at the genus level (Figure 4G, Supplemental File 8).

The transcriptome–microbiome correlation analysis results are shown in Figure 4H. *Candidatus_Arthromitus*, *Campylobacteraceae*, *Terrisporobacter*, and *Clostridium_sensu_stricto_1* were significantly correlated with the DEGs affected by Se. Specifically, the 4 genera were positively correlated with integrin subunit alpha 2b (*ITGA2B*), olfactory receptor 52B2-like (*OR52B2L*), steroidogenic acute regulatory protein (*STAR*),

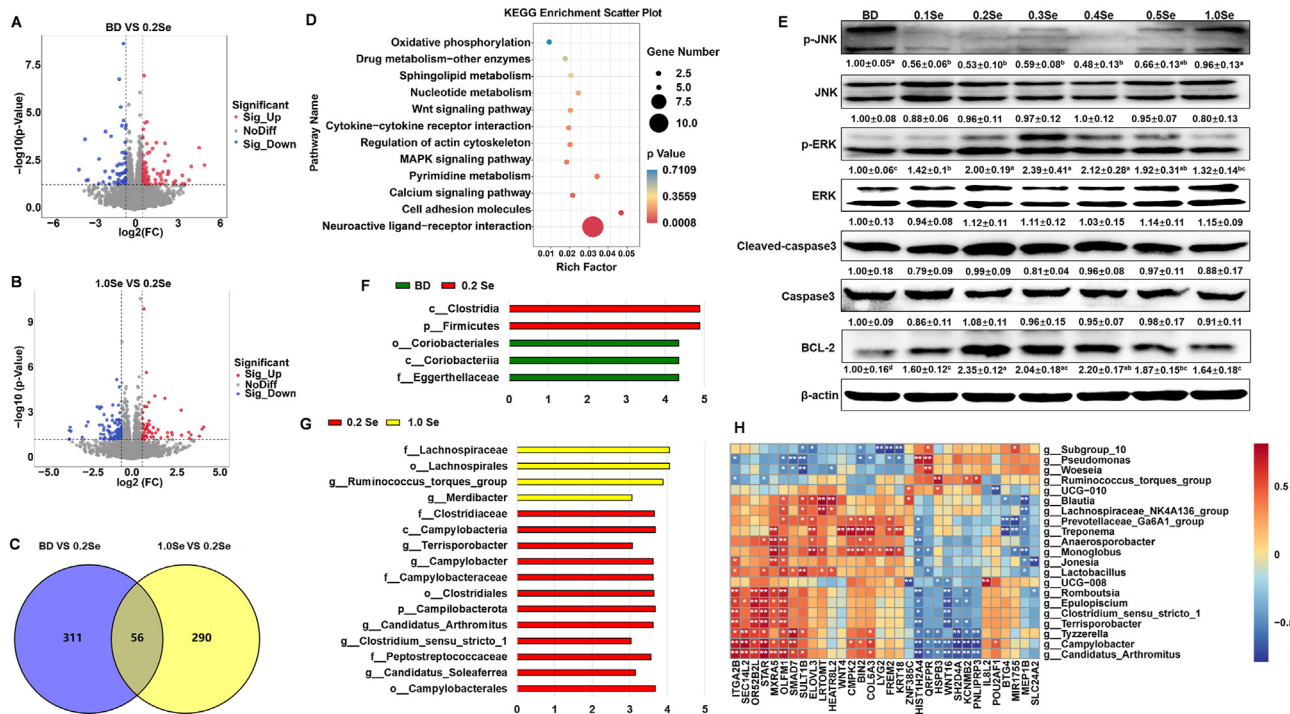


FIGURE 3. Effects of dietary Se dose on the testicular transcriptome and cecal microbiome of breeder roosters. Volcano plot of DEGs in the testis of the (A) BD and (B) 1.0Se compared with 0.2Se. (C) Venn diagram showing the unique and codifferentially expressed genes in the testis in BD and 1.0Se compared with 0.2Se. (D) Twelve enriched KEGG pathways of codifferentially expressed genes in the testis in BD and 1.0Se compared with 0.2Se. (E) Effects of dietary Se dose on the relative abundance of proteins involved in the MAPK/JNK/ERK signaling pathway in the testis of breeder roosters. Linear discriminant analysis effect size analysis of microbiota composition (F) between BD and 0.2Se, (G) between 1.0Se and 0.2Se. (H) Pearson's correlation coefficient analysis between bacteria and DEGs. The data are presented as the mean $\pm$ SE ($n = 6$). Labeled means without a common letter differ significantly, $P < 0.05$. Abbreviations: β -actin, beta-actin; BCL-2, B-cell lymphoma 2; BD, basal diet; DEG, differentially expressed gene; ERK, extracellular regulated kinase; KEGG, Kyoto Encyclopedia of Genes and Genomes; JNK, c-Jun-N-terminal kinase; Se, selenium; 0.1Se, 0.2Se, 0.3Se, 0.4Se, 0.5Se, and 1.0Se, BD supplemented with 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 mg Se/kg, respectively.

matrix remodeling associated 5 (*MXRA5*), and olfactomedin 1 (*OLFM1*) but negatively correlated with histone cluster 1, H2A, IV (*HIST1H2A4*), Wnt family member 16 (*WNT16*), SH2 domain containing 4A (*SH2D4A*), and potassium calcium-activated channel subfamily M regulatory β subunit 2 (*KCNMB2*). In addition, *Tyzzzeria* and *Epulopiscium*, belonging to *Firmicutes*, displayed a positive correlation with *ITGA2B*, *SEC14* like lipid binding 2 (*SEC14L2*), *OR52BL*, *STAR*, and *OLFM1* and a negative correlation with *HIST1H2A4*, *SH2D4A*, and *KCNMB2*.

The effects of different Se forms on body weight, sperm quality, sex hormones, and histopathology

There were no effects ($P \geq 0.05$) of the diets on the initial and final body weight of breeder roosters (Figure 4A, B). Compared with the BD, SeNa, SeY, and SeNPs increased ($P < 0.05$) sperm motility (14.8%–53.0%) and decreased ($P < 0.05$) sperm mortality (84.4%–99.8%) throughout the experiment (Figure 4C, D). Notably, SeNPs slightly increased ($P < 0.05$) sperm motility (5.3%–8.6%) throughout the experiment whereas SeY slightly increased ($P < 0.05$) sperm motility (4.5%) at week 7 compared with the SeNa (Figure 4C). In addition, SeNPs slightly decreased ($P < 0.05$) sperm mortality (12.8%) compared with SeY at week 9 (Figure 4D). Strikingly, the BD group had higher ($P < 0.05$) sperm density (19.5%–41.6%) than the other groups at weeks 5, 7, and/or 9 (Figure 4E). However, the serum testosterone, FSH, and LH concentrations were not different ($P \geq 0.05$) among the

treatment groups (Figure 4F–H). Moreover, there were histopathologic changes in the testis of breeder roosters fed the BD, including detachment of the spermatogenic epithelium from the basal membrane; disordered arrangement of spermatogenic epithelial cells; a reduction in the luminal cell density; and an increase in interstitial and spherical sperm cells, degenerated sperm, and remnants (Figure 4I). Meanwhile, the area of spermatogenic tubules in the BD group was reduced ($P < 0.05$) compared with that of the other groups (Figure 4J).

The effects of different Se forms on the Se concentration and redox parameters

Compared with the BD, SeNa, SeY, and SeNPs increased ($P < 0.05$) the serum and testis Se concentration by 72.8% to 107.7% and/or 28.7% to 134.7%, respectively (Figure 5A, F). Notably, SeNPs led to a higher ($P < 0.05$) serum Se concentration (23.0%–31.9%) compared with SeNa and SeY (Figure 5A, F). Compared with the BD, SeY and SeNPs reduced ($P < 0.05$) serum MDA concentration by 31.3% to 38.6%, as well as SeNa, SeY, and SeNPs reduced ($P < 0.05$) testicular MDA concentration by 36.5% to 36.7% (Figure 5B, G). Meanwhile, compared with the BD, SeNa increased ($P < 0.05$) serum total antioxidant capacity by 34.1% (Figure 5C). SeNPs improved ($P < 0.05$) testicular GPX activity (15.2%) compared with SeY (Figure 5I). Compared with the BD, dietary supplemented with the 3 forms of Se increased the serum TXNRD activity by 85.7% to 100% (Figure 5E), and

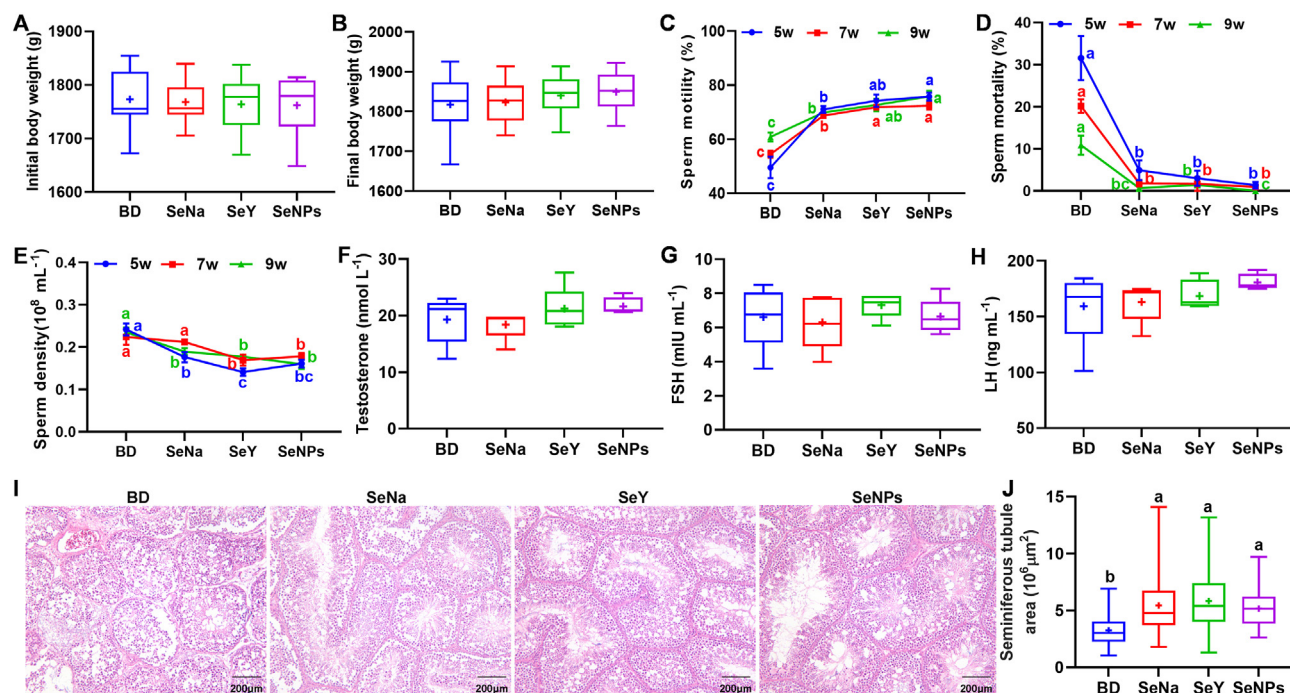


FIGURE 4. Effects of dietary Se forms on (A–B) body weight, (C–E) sperm quality, (F–H) serum hormone concentrations, and (I–J) testicular histopathology of breeder roosters. Data are presented as boxplots ranging from minimum to maximum or as mean $\pm$ SE ($n = 5$). Labeled means without a common letter differ significantly ($P < 0.05$). BD, base diet; Se, selenium; SeNa, SeY, and SeNPs, BD supplemented with 0.2 mg/kg sodium selenite, Se-enriched yeast, and Se-nanoparticles, respectively.

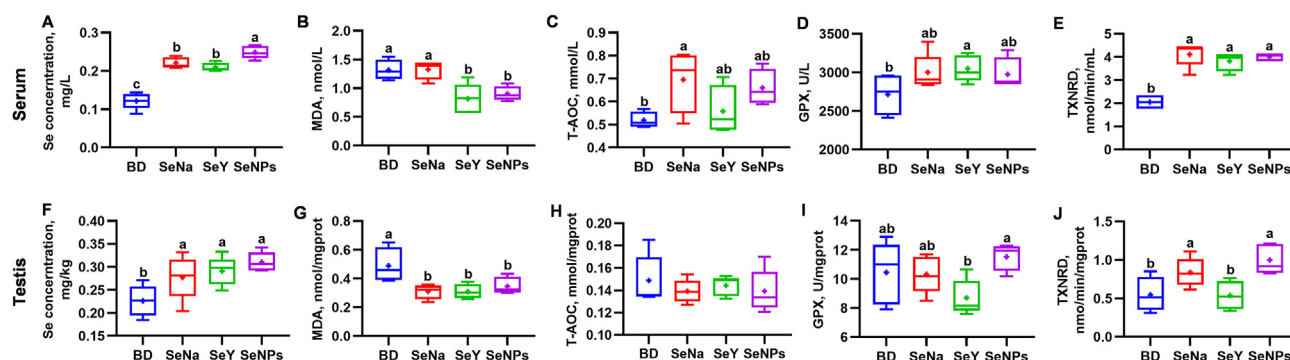


FIGURE 5. Effects of dietary Se forms on (A and F) Se concentration and (B–E), and (G–J) testicular redox index of breeder roosters. Data are presented as boxplots ranging from minimum to maximum ($n = 5$). Labeled means without a common letter differ significantly ($P < 0.05$). Abbreviations: BD, basal diet; GPX, glutathione peroxidase; MDA, malondialdehyde; Se, selenium; SeNa, SeY, and SeNPs, BD supplemented with 0.2 mg/kg sodium selenite, Se-enriched yeast, and Se-nanoparticles, respectively; T-AOC, total antioxidant capacity; TXNRD, thioredoxin reductase;

SeNPs increased ($P < 0.05$) testicular TXNRD activity by 82.8% to 86.1% compared with the BD and SeY (Figure 5J).

Production of selenoproteins and selected proteins involved in MAPK/JNK/ERK-mediated apoptosis and cell proliferation signaling

Compared with the BD, SeNPs increased ($P < 0.05$) the protein production of GPX1 and TXNRD1 and 3, whereas SeNa and SeY only increased ($P < 0.05$) the protein production of TXNRD3 (Figure 6A, B). SeNPs also led to higher ($P < 0.05$) GPX1 protein production than SeNa (Figure 6A, B). Meanwhile, SeNa and SeY decreased ($P < 0.05$) the protein production of JNK, and the 3 Se forms of supplementation decreased the

protein production of p-JNK in the testis compared with the BD (Figure 6C, D). Notably, SeNPs led to higher ($P < 0.05$) testicular p-ERK protein production than BD, SeNa, and SeY (Figure 6C, D). Compared with the BD, SeY and SeNPs increased ($P < 0.05$) BCL-2 protein production in the testis (Figure 6C, D). However, the protein production of ERK, cleaved-caspase 3, and caspase 3 were not affected ($P \geq 0.05$) by dietary Se.

Discussion

The most interesting finding from the current study is the quite narrow optimal range of dietary Se to ensure sperm quality of breeder roosters. Specifically, dietary supplementation with 0.2 mg Se/kg produced higher sperm motility and lower sperm

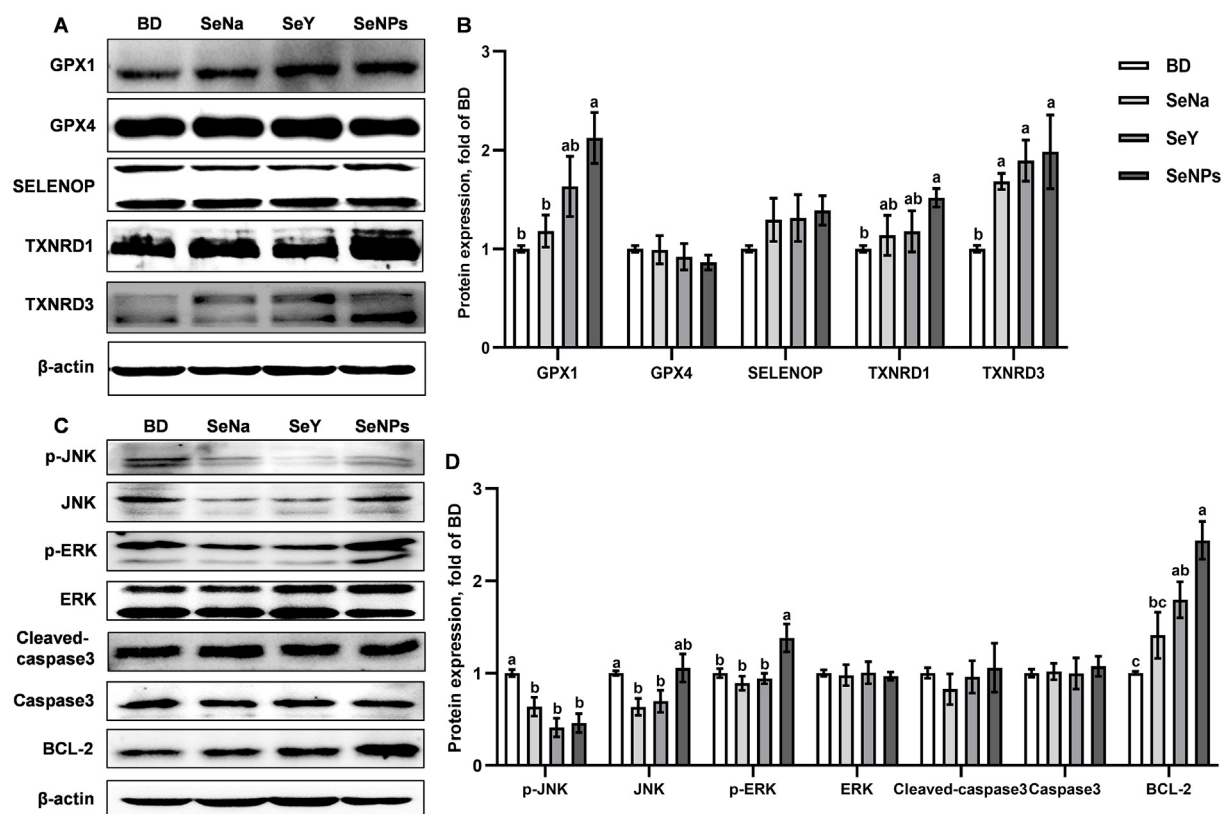


FIGURE 6. Effects of dietary Se forms on the production of selenoprotein and proteins involved in MAPK/JNK/ERK signaling pathway in the testis of breeder roosters. (A and C) Representative image of the protein bands. (B and D) Relative density of the protein bands. Data are presented as mean $\pm$ SE ($n = 3$ –4). Labeled means without a common letter differ significantly ($P < 0.05$). Abbreviations: BCL-2, B-cell lymphoma 2; BD, base diet; ERK, extracellular signal-regulated kinase; GPX, glutathione peroxidase; JNK, c-Jun-N-terminal kinase; Se, selenium; SeNa, SeY, and SeNPs, BD supplemented with 0.2 mg/kg sodium selenite, Se-enriched yeast, and Se-nanoparticles, respectively; SELENOP, selenoprotein P; TXNRD, thioredoxin reductase.

mortality compared with dietary Se deficiency (BD) or supplementation with 0.5 and 1.0 mg Se/kg at all the experimental time points and compared with dietary supplementation with 0.1, 0.3, and 0.4 mg Se/kg at some of the experimental time points. These findings are similar to previous studies that have shown dietary Se deficiency or supplementation >0.5 mg/kg reduced spermatogenesis and sperm quality in mice, rats, and roosters [18,20,21,34]. Furthermore, our study provides a more precise estimate of the optimal dietary Se concentrations for breeder roosters [18,35,36]. Strikingly, dietary Se deficiency (BD) or supplementation with 1.0 mg Se/kg led to higher sperm density than dietary supplementation with 0.2 mg Se/kg at weeks 5 and/or 9. These outcomes seem to conflict with those of previous studies that have reported Se deficiency or excess reduce sperm quality [18,19,35,36]. This discrepancy could be due to the different experimental durations, diets, species, and ages of the animals [19,37]. It has been well documented that FSH, a gonadotropin, acts on Sertoli cells in concert with testosterone to play an important role in spermatogenesis [38]. In this study, dietary Se deficiency (BD) increased serum testosterone and FSH concentrations, whereas supplementation with 1.0 mg Se/kg reduced serum testosterone more than supplementation with 0.2 and/or 0.3 mg Se/kg. The dysregulation of these pivotal sex hormones could explain why the extreme concentrations of Se tested in the current study decreased sperm quality. Formation of high-quality sperm requires normal

seminiferous tubules and Se deficiency altered testicular morphology and promotes seminiferous tubule degeneration [39]. In the present study, dietary supplementation with 0.2 to 0.4 mg Se/kg led to better testicular histology than dietary Se deficiency (BD) or supplementation with 0.1, 0.5, and 1.0 mg Se/kg. Taken together, our study reveals that there is a very narrow optimal range of dietary Se, 0.25 to 0.35 mg Se/kg (considering that the BD contained 0.06 mg Se/kg), for the reproductive system of Jingbai laying breeder roosters. These outcomes indicated that the male reproductive system might be very sensitive to Se ingestion. Nevertheless, further studies are needed to measure the optimal doses of dietary Se in the other species of male breeders. Because the regulatory limits for Se in feed is 0.5 mg/kg around the world [40,41], the optimal Se dose for breeder roosters used in production should be determined.

Oxidative stress can impair the integrity of DNA, proteins, and lipids in testicular and sperm cells, thus resulting in testicular damage and decreased sperm quality [42]. Notably, the current study showed that roosters exposed to Se deficiency (BD) or dietary supplementation with 0.1, 0.5, or 1.0 mg Se/kg experienced oxidative stress, as indicated by the increased DNA damage (8-OHdG) in the testis, compared with dietary supplementation with 0.2, 0.3, and/or 0.4 mg Se/kg. Meanwhile, dietary Se deficiency (BD) reduced the activity of the redox enzyme TXNRD in the testis compared with the other groups. These outcomes are similar to previous studies that have

reported both Se deficiency and excess induce oxidative stress in the testis. Oxidative stress is one of the primary ways by which suboptimal Se concentrations induce adverse effects on male reproductive functions [21,34]. Se deficiency decreases the production of redox selenoproteins, and Se excess induces the production of superoxide radicals [43–45], thus resulting in oxidative stress in different animals [21,43,46]. Notably, we demonstrated that there is a quite narrow optimal range of dietary Se, 0.2–0.4 mg Se/kg that maintains the redox balance in the testis of Jingbai laying breeder roosters.

The functions of Se in poultry are mostly exerted via 25 selenoproteins [43]. Thus far, 6 selenoproteins, GPX1, GPX3, GPX4, GPX5, TXNRD3, and SELENOP, have been reported to support male reproductive function mainly through redox regulation [40–46]. In this study, BD downregulated the expression of 9 selenoprotein genes, *SELENOK*, *SELENOP*, *MSRB1*, *GPX3*, *SELENOH*, *SELENOO*, *SELENOP2*, *SELENOU*, and *TXNRD3*, in the testis compared with supplementation with 0.2, 0.3, and/or 0.4 mg Se/kg. Because these selenoproteins play roles in redox control, their downregulation in the BD group contributes to Se deficiency-induced oxidative stress and testicular injury [43,45–53]. Dietary supplementation with 0.5 and 1.0 mg Se/kg also upregulated the expression of 10 antioxidant selenoproteins, *DIO1*, *MSRB1*, *GPX3*, *SELENOH*, *SELENOO*, *SELENOP2*, *SELENOU*, *TXNRD2*, and *TXNRD3* [47–53]. However, these changes could not overcome the excessive Se intake-induced testicular oxidative stress and damage. Furthermore, the testicular expression of the 8 remaining selenoprotein genes (*DIO2*, *GPX1*, *GPX2*, *SELENOF*, *SELENOI*, *SELENON*, *SELENOT*, and *SEPHS2*) failed to respond to the dietary Se concentration, perhaps because they are too important to be altered or they are unimportant and insensitive to dietary Se. Nevertheless, the exact roles and mechanisms of the selenoproteins in male reproduction need to be further explored in specific gene knockout animal models.

To better understand the molecular biology of Se on the reproductive function of roosters, we analyzed testicular transcriptomic and cecal microbiomic changes. Specifically, compared with the 0.2 mg Se/kg group, 367 and 346 DEGs were altered by dietary Se deficiency and excess, respectively, in the testis of roosters. Notably, the DEGs affected by both dietary Se deficiency and excess were primarily enriched in the neuroactive ligand–receptor interaction, cell adhesion molecules, the calcium signaling pathway, and the MAPK signaling pathway. Although these signaling pathways have been reported to be involved in male reproduction by regulating reproductive endocrine hormone homeostasis and spermatogenesis [54,55], their potential involvement in Se-mediated reproductive functions is a novel finding of our current study. Furthermore, oxidative stress-regulated MAPK/JNK/ERK signaling plays an important role in sperm development and testicular health by regulating cell proliferation, differentiation, apoptosis, and environmental responses [54–56]. Specifically, JNK is an important enforcer of the MAPK signaling pathway in response to stress and regulation of inflammation and apoptosis, and ERK is an important determinant of the MAPK signaling pathway in regulating cell proliferation and differentiation; upregulation of p-JNK can induce apoptosis, whereas upregulation of p-ERK can promote cell proliferation [57,58]. In the present study, dietary Se deficiency (BD) and supplementation with at 0.1, 0.5, and/or 1.0 mg Se/kg

upregulated p-JNK and downregulated p-ERK and its downstream anti-apoptosis protein BCL-2 in the testis compared with dietary supplementation with 0.2–0.4 mg Se/kg. These outcomes indicate that Se deficiency and excess might induce apoptosis and inhibit cell proliferation, which may be attributed to the testicular injury and reduced sperm quality we observed.

The gut microbiota affects testicular function by regulating the host's metabolism [59]. Some *Firmicutes* produce spermine, which exerts an antioxidant effect by protecting against oxidative stress-induced testicular injury and maintaining reproductive homeostasis in males [60–63]. In the present study, Se deficiency and/or excess reduced the abundance of *Firmicutes*, which might underlie the testicular damage and sperm quality decline induced by the 2 extreme Se doses [61–63]. Previous studies have reported that short-chain fatty acids, acetic acid and butyric acid, inhibited the apoptosis and inflammation induced by oxidative stress in testis and increased sperm quality [64,65]. *Terrisporobacter* and *Clostridium_sensu_stricto-1* can produce short-chain fatty acids, which can protect against oxidative stress-induced defective spermatogenesis, hypotestosteronemia, and other testicular disorders [66]. However, Se excess led to a decrease in the abundance of *Terrisporobacter* and *Clostridium_sensu_stricto-1*, which could contribute to the reproductive dysfunction induced by oxidative stress observed in the present study. Moreover, the gene–microbiota correlation analysis revealed that Se deficiency and excess dysregulated the abundance of bacterial (*Terrisporobacter* and *Clostridium_sensu_stricto-1*) and MAPK signaling pathway-related gene expression (*ITGA2B*, *MXRA5*, *SMAD7*, and *WNT16*), which might regulate inflammation, cell proliferation, and differentiation [67–72]. Taken together, these findings suggest that Se deficiency or excess disrupts the gut microbiota, leads to testicular transcriptome dysregulation, and reduces reproductive health in roosters.

Another interesting finding from this study was that SeNPs displayed a better ability to improve the sperm quality of the roosters compared with SeNa and SeY. Specifically, SeNPs produced higher sperm motility throughout the experiment compared with SeNa and decreased sperm mortality at week 9 compared with SeY. These results might be because SeNPs increased Se deposition in the serum and improved the antioxidant capacity, as indicated by the reduced serum MDA concentrations and increased testicular GPX and TXNRD activities and GPX1 protein production compared with SeNa and/or SeY. These outcomes are similar to previous studies that reported that compared with SeNa and SeY, SeNPs were absorbed and utilized more effectively by the intestines, increased the expression of selenoenzymes (such as GPX and TXNRD), and then scavenged reactive oxygen species [24,27,73,74]. Furthermore, SeNPs increased the testicular protein expression of ERK and BCL-2, which are related to oxidative stress-regulated cell proliferation and anti-apoptotic signaling, more than SeNa and/or SeY [56–58]. This might explain why dietary supplementation with SeNPs led to better sperm quality and testis health in the roosters than the other 2 Se forms in the current study.

In summary, this study showed that there is a U-shaped relationship between Se intake and reproductive system function in Jingbai laying breeder roosters (Figure 7). Our results revealed that the optimal safe dose of Se for Jingbai laying breeder roosters is 0.25–0.35 mg Se/kg. Furthermore, dietary Se

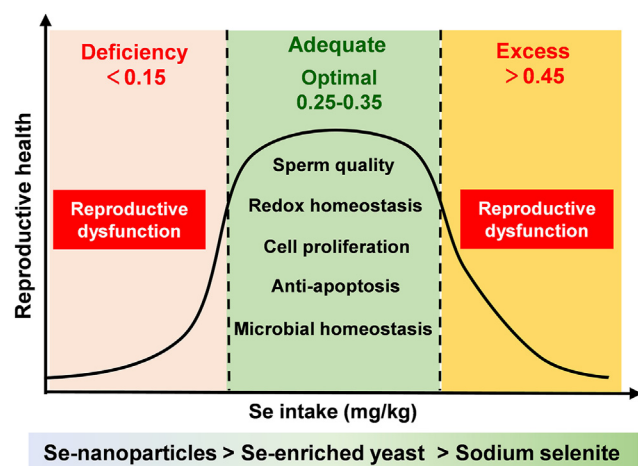


FIGURE 7. An illustration of the beneficial and harmful effects of various dietary selenium doses and forms on the reproductive health of breeder roosters.

deficiency and excess impaired the reproductive system, causing histopathologic lesions and decreasing sperm quality in the testis of the roosters. These changes were associated with impaired microbiota and redox homeostasis and dysregulated of MAPK/JNK/ERK-mediated apoptosis and cell proliferation signaling. Additionally, dietary supplementation with SeNPs improved the reproductive health of the roosters a bit more than SeNa and SeY. This improvement was associated with the higher bioavailability and antioxidant activity of SeNPs. These results provide guidance for the application of Se in animal production and can serve as reference value for the improvement of male reproductive health.

Author contributions

The authors' responsibilities were as follows – L-HS, XGL, GZ: designed the research; Y-QY, ML, Ze-Jing Xu, Zi-Juan Xu, Y-XH, X-ML, C-JC, J-CY: conducted the experiments and statistical analysis; ML, Y-QY: performed bioinformatics analysis and visualized the data; ML, Y-QY, L-HS: wrote the manuscript; L-HS: had primary responsibility for the final content; and all authors: read and approved the final manuscript.

Conflict of interest

The authors report no conflicts of interest.

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Data availability

Data described in the manuscript will be made available upon request pending approval.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tjn.2023.12.021>.

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