Dietary supplementation with selenium-enriched earthworm powder improves antioxidative ability and immunity of laying hens

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ABSTRACT Selenium (**Se**) has been recognized as an essential dietary nutrient for decades, and organic Se sources rather than inorganic ones are increasingly advocated as Se supplements. Earthworms have been studied as a feed additive and animal protein source for many yr. The aim of this study was to evaluate the effect of Se-enriched earthworm powder (**SEP**) on the antioxidative ability and immunity of laying hens. A total of 120 27-wk-old laying hens were randomly divided into 4 groups (30 hens per group). Laying hens were fed diets supplemented with SEP having 0, 0.5, or 1 mg/kg of Se or with earthworm powder alone. After 5 wk of supplementation, serum from the hens was tested for nutritional components (protein, globulin, albumin, triglycerides, total cholesterol, and glucose), antioxidative properties (glutathione peroxidase, superoxide dismutase, catalase, and nitric oxide), and immune responses (lysozymes, immunoglobulin G, IL-2, and interferon gamma). We found that SEP with 1.0 mg/kg of Se upregulated the hens' total protein, albumin, glutathione peroxidase, superoxide dismutase, IgG, and IL-2 and downregulated triglycerides, total cholesterol, glucose, and nitric oxide. These results indicate that SEP improves antioxidative levels and immune function of laying hens, indicating potential benefit from use of SEP as a feed additive in the poultry industry.

Key words: selenium-enriched earthworm powder, laying hen, antioxidative property, immune response

2020 Poultry Science 99:5344–5349
https://doi.org/10.1016/j.psj.2020.07.030

INTRODUCTION

Selenium (**Se**) is an essential trace element for animal health, as it is involved in many biological functions, including those related to antiaging (Papp et al., 2010), reproduction (Harrison et al., 1984), neurobiology (Schweizer et al., 2004), muscle metabolism (Brown and Arthur, 2001), chemoprevention (Combs et al., 1998), and immune functions (Hoffmann and Berry, 2008). The beneficial and protective properties of Se are mainly owing to its antioxidative activities against carcinogenic factors and free radicals (Kieliszek and Błażejak, 2016). Selenium deficiency has been shown to be associated with reduced productivity and dysfunctional reproductivity in the poultry industry (Surai, 2002). Thus, support has grown for dietary supplementation with Se as means of maintaining poultry health and an effective way to increase the Se content of carcass meat and eggs (Federal Register, 2002).

Sodium selenite, one of the inorganic forms of Se, has been widely used as an Se source in major feed ingredients over the last 50 yr (Surai, 2006). However, the bioavailability and safety of inorganic Se are generally lower than those of organic Se (Daniels, 1996). For example, organic forms are more effective than inorganic Se from sodium selenite in increasing blood Se concentrations and glutathione peroxidase (**GSH-Px**) activities in lambs and in increasing Se content in the carcass meat of broilers (Mahan and Parrett, 1996). Organic Se sources include microorganisms and plants that have the ability to transport and accumulate organic Se from selenate or selenite. Microorganism-derived Se sources include Se-enriched yeast, probiotics, and algae (Skrivan et al., 2006). Plant-derived Se sources include Se-enriched bean sprouts (Chinrasri et al., 2009), broccoli (Bañuelos et al. 2015), garlic (Yang, 2002), and cabbage (Seo et al., 2008).

The findings cited previously demonstrate that microorganism-derived Se and plant-derived Se are good organic Se sources for the poultry industry.

Received April 27, 2020.
Accepted July 6, 2020.

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However, there is little reporting on organic Se from animal sources. Hu extracted Se-enriched amino acids and selenoprotein from Ziyang silkworm cocoons and studied the effects of Se-enriched amino acids on hepatoma cells (Hu et al., 2004; Liu et al., 2004). Hall et al. (2014) demonstrated that Se-enriched yeast supplementation improved antioxidative activity and immune responses without impacting other micronutrients or energy status. Similarly, selenoprotein W has an important protective function against H_2O_2-induced oxidative damage (Han et al., 2012). Previous studies have reported that organic Se can be accumulated in earthworms and can reach concentrations as high as 332.5 mg/kg of Se dry weight (Liu et al., 2001; Sun et al., 2014). However, the effects of Se-enriched earthworms on poultry production have been completely unknown. In this study, we investigated the effects of Se-enriched earthworm power (**SEP**) on laying hens, including its effects on antioxidative activities and immune responses.

MATERIALS AND METHODS

The Production of Earthworm Powder

Earthworms (*Eisenia fetida*) were fed with cow dung and divided into two groups. Sodium selenite at 60 mg/kg was added to the feed of Se-enriched earthworm group (SEP group), and the same amount of sodium chloride was added to the feed of non–Se-enriched earthworm group (control group). After 60 D of feeding, earthworms and worm casts were collected, freeze-dried, and ground into powder (in a weight ratio of 2:3). The Se content of the SEP was 57 mg/kg, whereas the concentration of Se in the control group was 5.85 mg/kg. To ensure all treatment groups got the same amount of exogenous supplement, we first mixed the SEP with powdered earthworms without Se enrichment to a given amount and then added the same amount (1.8%) of supplement into the hens' basal diet. The diet concentrations of exogenous-source Se (not including the basal Se content) for the treatment groups were 0 mg/kg for the T2 group (1.8% control earthworm powder addition), 0.5 mg/kg for the T3 group (1.8% SEP addition), and 1 mg/kg for the T4 group (1.8% SEP addition).

Main Reagents and Test Instruments

The kits were obtained from Nanjing Jiancheng Bioengineering Institute for biomedical research, including GSH-Px, superoxide dismutase (**SOD**), catalase, nitric oxide, lysozyme, IgG, IL-2, and interferon gamma. The instruments applied in our test included a TU-1810 UV and Visible Spectrophotometer (Beijing Purkinje General Instrument Co. Ltd.), HH-2 thermostatic water bath with digital display (Shanghai Techeng mechanical equipment Co. Ltd.), IEC Micro microcentrifuge and Mk3 ELISA (Thermo company), AU2700 automatic biochemical analyzer (Olympus Company, Japan), 96-hole MPP oscillator (Beijing Jiayuan Xingye Technology Company), and incubator (Beijing Fuyi Electrical Equipment Company).

Experimental Procedures and Dietary Treatments

One hundred twenty adult laying hens (27 wk of age; Lohmann type) were randomly assigned to 4 groups (with three replicates of 10 hens for each group). All hens were acclimated to a basal diet for 3 wk. No significant difference in feed intake was observed among experimental individuals before treatment. Hens were fed diets supplemented without supplementation (basal diet, T1 group), earthworm powder alone (1.8% added to the basal diet, T2 group), 0.5 mg Se per kg SEP (T3 group), or 1 mg Se per kg SEP (T4 group) three times per day at 6 am, 11 am, and 6 pm for 5 wk. The basal diet (Table 1) was formulated to meet the nutrient requirements of hens (National Research Council, 1971) without Se supplementation. The hens were housed under a 16L:8D cycle at a constant temperature (20 ± 4 °C), and the RH changed between 60% and 78% during the experiment. All animal handling protocols used with experimental individuals were reviewed and approved by the Institutional Animal Care and Use Committee at China Agricultural University.

Table 1. Ingredients and nutrient level of control diets.

Ingredients	Percentage (%)	Nutrient level	Content
Corn	62.50	CP/%	16.70
Soybean Meal	20.00	Crude Fibre/%	3.35
Limestone	8.50	Lysine/%	0.75
Bran	5.00	Methionine/%	0.39
Vitamin–mineral premix[a]	4.00	Cystine/%	0.32
		Digestible energy(ME)/kJ·kg^{-1}	11.43
		Phosphorus/%	0.74
Total	100	Calcium/%	3.50
		Selenium/mg·kg^{-1}	0.012

Note: The nutrient content in feed formulation is theoretical calculation values.

[a]Vitamin-mineral premix provide (per kg diet): 25,300 IU vitamin A, 7,000 IU vitamin D $_3$, 270 IU vitamin E, 45 mg vitamin K_3, 32 mg vitamin B_1, 110 mg vitamin B_2, 30 mg vitamin B_6, 0.6 mg vitamin B_{12}, 40 mg niacin, 13 mg pantothenic acid, 13 mg folic acid, 0.8 mg biotin, 100 mg choline chloride, 60 mg Fe,1.4 mg Cu, 60 mg Mn,13 mg Zn, 0.012 mg Se, 0.098 mg I.

Table 2. Effects of selenium-enriched earthworms power on the biochemical index.

Dietary treatments	Protein(g/L)	Globulin(g/L)	Albumin(g/L)	Triglyceride (g/L)	Total cholesterol (g/L)	Glucose (g/L)
T1	49.90 ± 1.27^{c}	29.75 ± 1.34^{b}	20.15 ± 0.07^{b}	20.46 ± 0.11^{a}	3.04 ± 0.08^{a}	15.07 ± 1.08^{a}
T2	53.90 ± 0.57^{b,c}	33.20 ± 0.42^{a,b}	20.70 ± 0.14^{b}	18.16 ± 0.37^{a,b}	2.89 ± 0.14^{a,b}	14.22 ± 0.04^{a,b}
T3	58.50 ± 0.42^{a,b}	37.45 ± 0.78^{a}	21.05 ± 0.35^{b}	17.14 ± 0.47^{a,b}	2.81 ± 0.35^{b}	14.17 ± 0.30^{a,b}
T4	61.95 ± 4.88^{a}	39.15 ± 4.60^{a}	22.80 ± 0.28^{a}	15.22 ± 3.42^{b}	2.75 ± 0.01^{b}	13.60 ± 0.17^{b}

$^{a–c}$Different letters in the same column indicate significant difference ($P < 0.05$; ANOVA and Turkey's test).

Abbreviations: T1, control group (basal diet); T2, control diet earthworm powder alone (no Se enrichment); T3, control diet plus 0.5 mg Se/kg from selenium-enriched earthworms powder (SEP); T4, control diet plus 1.0 mg Se/kg from SEP, respectively.

Sampling and Biochemistry Analysis

Samples from the diets were randomly collected for the determination of chemical composition at the end of the experiment. On the 35th D of the experiment, the hens fasted for 3 h and were then electrically stunned. Then, 5-mL blood samples were obtained into sterile test tubes from the brachial vein of the wing. The samples were centrifuged at 3,000 r/min for 20 min, and the supernatants were harvested, aliquoted, and stored at -80 °C before testing.

The levels of serum protein, globulin, albumin, triglycerides, total cholesterol, and glucose were tested using an AU2700 automatic biochemical analyzer. The activity of GSH-Px and SOD were investigated by means of dinitro benzoic acid, xanthine oxidase, ammonium molybdate colorimetry, and nitrile reductase. The instrument used was a TU-1810 UV and visible spectrophotometer. The levels of lysozyme, IgG, IL-2, and interferon gamma were measured by immunoturbidimetry and ELISA.

Statistical Analysis

Statistical analyses were conducted as previously described (Sun et al., 2017). Each animal was considered as an experimental unit. The experimental data were analyzed using one-way ANOVA and represented with average value ± SD (mean ± SD). Statistical analysis was performed using Prism 6 software (GraphPad Software, Inc., La Jolla, CA). The differences among the groups were determined using Duncan's multiple range tests. Significance was accepted at $P < 0.05$.

RESULTS

The Impacts of SEP on Serum Biochemistry Profile

To determine the effects of SEP on the health status of laying hens, we tested the biochemistry profile of chicken serum, including the content of protein, globulin, albumin, triglyceride, total cholesterol, and glucose (Table 2). Of note in this study, earthworm powder alone (T2 treatment) increased protein, globulin, and albumin by 8.02%, 8.53%, and 11.60%, respectively, but decreased triglycerides, cholesterol, and glucose by 11.24%, 5.61%, and 4.78%, respectively, suggesting that earthworms could be a potential dietary substitute for laying hens. Selenium-enriched earthworm powder with 1.0 mg Se per kg (T4) positively and significantly affected the protein content in serum in a dose-dependent manner, possibly owing to the upregulation of globulin and albumin, as compared with the serum protein content from hens fed the control diet (T1) and the control diet plus earthworm powder alone (T2) ($P < 0.05$). Selenium-enriched earthworm powder with 1.0 mg Se per kg (T4) significantly reduced the concentration of triglycerides, total cholesterol, and glucose compared with the control diet (T1) ($P < 0.05$). Selenium-enriched earthworm powder with 0.5 mg Se per kg (T3) significantly increased the concentration of globulin and decrease triglycerides compared with the control diet (T1) ($P < 0.05$).

The Impacts of SEP on Serum Antioxidative Capacity

Selenium is known for its antioxidant properties. The activity of antioxidative enzymes including GSH-Px, SOD, and catalase were tested to evaluate the antioxidative role of SEP in laying hens (Table 3). Selenium-enriched earthworm powder with 1.0 mg Se per kg (T4) significantly increased the activity of antioxidative enzymes (GSH-Px and SOD), whereas it downregulated the generation of nitric oxide in serum compared with the control diet (T1) and the control diet plus earthworm powder alone (T2) ($P < 0.05$). Selenium-enriched earthworm powder with 0.5 mg Se per kg

Table 3. Effects of selenium-enriched earthworms power on the antioxidant ability.

Dietary treatments	Glutathione peroxide Enzyme(U·mL^{-1})	Superoxide dismutase (U·mL^{-1})	Catalase (U·mL^{-1})	Nitric Oxide(μM·L^{-1})
T1	148.32 ± 6.34^{b}	100.06 ± 9.44^{b}	2.09 ± 0.64^{b}	30.03 ± 0.95^{a}
T2	150.26 ± 7.90^{b}	103.37 ± 2.31^{b}	2.37 ± 0.30^{a,b}	26.87 ± 1.74^{a,b}
T3	188.08 ± 9.17^{a,b}	106.79 ± 2.04^{a,b}	2.25 ± 0.85^{a,b}	24.36 ± 9.75^{a,b}
T4	208.45 ± 10.25^{a}	107.24 ± 1.62^{a}	2.90 ± 0.69^{a}	22.03 ± 3.28^{b}

$^{a–b}$Different letters in the same column indicate significant difference ($P < 0.05$; ANOVA and Turkey's test).

Abbreviations: T1, control group (basal diet); T2, control diet earthworm powder alone (no Se enrichment); T3, control diet plus 0.5 mg Se/kg from selenium-enriched earthworms powder (SEP); T4, control diet plus 1.0 mg Se/kg from SEP, respectively.

Table 4. Effects of selenium-enriched earthworms power on the immunity.

Dietary treatments	Lysozyme/(μg·mL^{-1})	IgG/(μg·mL^{-1})	IL-2/(ng·mL^{-1})	Interferon-gamma/(ng·mL^{-1})
T1	6.85 ± 0.81^{b}	4.98 ± 0.55^{b}	2.17 ± 0.26^{b}	70.12 ± 11.56^{b}
T2	7.37 ± 0.86^{a,b}	5.42 ± 0.84^{b}	2.45 ± 0.33^{b}	80.30 ± 3.99^{a,b}
T3	8.13 ± 0.77^{a,b}	7.18 ± 0.95^{a}	3.20 ± 0.31^{a}	99.08 ± 8.10^{a}
T4	8.66 ± 1.07^{a}	9.08 ± 0.83^{a}	3.66 ± 0.06^{a}	105.50 ± 8.87^{a}

$^{a–b}$Different letters in the same column indicate significant difference ($P < 0.05$; ANOVA and Turkey's test).

Abbreviations: T1, control group (basal diet); T2, control diet earthworm powder alone (no Se enrichment); T3, control diet plus 0.5 mg Se/kg from selenium-enriched earthworms powder (SEP); T4, control diet plus 1.0 mg Se/kg from SEP.

(T3) significantly increased the activity of GSH-Px and SOD and decreased the generation of nitric oxide in serum compared with the control diet (T1) ($P < 0.05$). Earthworm powder without Se pretreatment had the potential to eliminate free radicals by activating antioxidative enzymes.

The Impacts of SEP on the Immune Response

It has been reported that Se supplements improve the immune response, whereas Se deprivation causes an impaired immune response (Marsh et al., 1981; Xu and Tian, 2015). In this study, we found that lysozyme, IgG, IL-2, and interferon gamma levels did not respond to treatment with the control diet plus earthworm control powder (T2) ($P > 0.05$), whereas they were significantly increased in serum in response to treatment with SEP with 0.5 mg Se per kg (T3) or SEP with 1.0 mg Se per kg (T4) when compared with treatment with the control diet (T1) or the control diet plus earthworm control powder (T2) ($P < 0.05$), suggesting that SEP improved the immune response of laying hens (Table 4).

DISCUSSION

Selenium supplementation is indispensable for hens' performance in regions where Se in the environment is low, which can be reflected in serum protein content (Payne and Southern, 2005). We studied the effects of SEP on hen serum in this study. Our results show that SEP can improve serum protein content. This may be because the metabolism and utilization of protein and amino acids are greater in response to Se supplementation (Hao et al., 2010). It would also be an interesting study to measure the Se contents in both plasma and tissue to test the dose responses in hens.

Albumin acts as a nutrition carrier, and globulin is one of the immune proteins. The ratio of albumin to globulin in serum could be used as an important indicator of animal nutrition and immunity performance (Bunchasak et al., 2005). Serum albumin plays an important role in keeping the plasma colloid osmotic pressure, adjusting the hydrodynamic equilibrium between the organs and the blood vessels (Kaneko, 1997). The downregulation of albumin levels could be a disease indicator related to dysfunction of the liver, heart, or kidneys (Honda et al., 2006). Selenium-enriched earthworm powder significantly increased the content of serum albumin, which might suggest a method to alleviate these diseases.

Serum total cholesterol and triglycerides are two commonly used indexes in reflecting body lipid metabolism, abnormal elevation of which is a reflection of a lipid metabolism disturbance. Cows fed an Se-enriched yeast supplement during the last 8 wk of gestation had lower serum cholesterol concentrations compared with control cows (Hall et al., 2014). Selenium-enriched earthworm powder with 1 mg Se per kg decreased the content of serum total cholesterol and triglycerides, providing a clue that earthworm powder enriched with Se plays a significant role in adjusting hens' body lipid metabolism and reducing blood fat.

Oxidative stress is a condition that refers to an imbalance between levels of reactive oxidative species and their antioxidants. Oxidative stress can come from exogenous sources (such as in pathogen-induced or chemically induced stress) or endogenous sources, such as endogenous metabolism from mitochondria, peroxisomes, cytoplasm, or plasma membrane (Biller and Takahashi, 2018). The antioxidant defense system of the organism mainly uses GSH-Px, SOD, and catalase for removal of excess free radicals, namely, O^{-}, H_2O_2, and ROO^{-}. Selenium deficiency could lead to the decline of antioxidative activities and an increase in free radicals, causing damage to lipid peroxidation, proteins, and nucleic acids, thereby inducing pancreatic necrosis in birds and animal reproductive dysfunction (Wu et al., 2010). Our results found that SEP improved the serum levels of GSH-Px and SOD, whereas earthworm powder alone failed to enhance the activity of these antioxidative enzymes, indicating that Se might play a major role in promoting the generation of GSH-Px and SOD. Similarly, Wang et al. (2011) found that antioxidant status greatly improved in broilers in an L-Se-Met–treated group, which was illuminated by the increased glutathione concentration in serum. Furthermore, GSH-Px activity was higher in broilers supplemented with hydroponically produced Se-enriched kale sprouts than in those supplemented with Se from sodium selenite and Se-enriched yeast (Chantiratikul et al., 2015). The GSH-Px levels of weaned piglets in a 0.5 mg/kg nanoselenium group were significantly higher than those in the control group (Li et al., 2018). Supplementation of Se can also improve serum antioxidative status in the blood of growing male goats (Shi et al., 2011). Administration of Se polysaccharide at levels of 0.30, 0.45, and 0.60 mg/kg significantly increased activities of serum GSH-Px and SOD in weaning piglets (Liu et al., 2013). In a 0.3 mg/kg DL-

selenomethionine group, the activities of GSH-Px and SOD in broiler serum increased significantly (Zhang et al., 2010). The addition of 0.25 or 0.5 mg/kg Se-enriched yeast in the diet significantly improved layer serum GSH-Px activity ($P < 0.01$) and the trend of total superoxide dismutase activity (Gao et al., 2006). With a diet with 0.1–0.5 mg/kg Se (yeast Se), the activities of total antioxidant capacity, GSH-Px, and total superoxide dismutase in the serum of geese were increased significantly in different feeding time, and the optimal supplemental level of Se was 0.3 mg/kg (Wang et al., 2009).

Dietary Se incorporates into selenoproteins that are important for immunity initiation and the synthesis of antioxidant enzymes (Huang et al., 2012). One study investigated the effects of sodium selenite on the mRNA expression of glutathione peroxidase 1, an important antioxidant enzyme (Ren et al., 2012). Ren et al. found that splenocytes without any stimulation start to synthesize the transcriptome of antioxidant enzymes in response to Se treatment. Similarly, we found in our study that Se addition alone causes immune activation. Lysozyme participates in a variety of immune responses (Saurabh and Sahoo, 2008). For example, it repairs and regenerates cells during the process of inflammation, and it has the vital function of maintaining physiological balance. In this study, SEP significantly increased the level of lysozyme in the serum, suggesting its higher activities of phagocytes. IgG, mainly involved in the humoral immune response, is one of the active constituents in mediating humoral immunity to resist infections, participating in the process of antisepsis, antineoplastic, resistant parasites, and some abnormal reaction (Vaezirad et al., 2018). In this experiment, SEP significantly increased the layer serum content of IgG, in line with observations of increased serum IgG in piglets after supplementation with 0.3 mg/kg of Se-enriched yeast (Xu et al., 2018). Furthermore, a high concentration of added selenomethionine can stimulate lymph cells to produce more Ig when the lymph cells are cultivated outside the body (Wu et al., 2015). Yoon and McMillan found that 0.3 mg/kg yeast Se and sodium selenite can improve the content of serum IgG of newborn piglets (Yoon and McMillan, 2006).

IL-2, one of the cellular immune responses, is an important immune regulatory factor for adjusting the growth, differentiation, and proliferation of cells and reduces viral or bacterial infection. Selenium-enriched earthworm powder had the ability to significantly improve the level of serum IL-2 compared with that in the control group. Selenium supplementation with both sodium selenite and Se-enriched probiotics can significantly increase the serum IL-2 level of chickens (Pan et al., 2011). Moreover, the serum concentrations of IL-2 also showed positive responses in chicken fed with Se-enriched exopolysaccharides (Liu et al., 2013). Interferon gamma, produced by T cells, has multiple effects in resisting tumors, bacteria, viruses, and parasites and plays an important role in helping mammals and birds fight pathogen infections (Harada et al., 1998). We found that SEP positively stimulated interferon gamma levels in serum after the hens were fed for 35 D. This effect was similar to the generation of interferon gamma seen when human peripheral blood lymphocytes were cultivated with Se (Watson and Leonard, 1986).

CONCLUSION

In this study, SEP was served as a feed additive to laying hens. The effects of SEP on nutrition metabolism, antioxidant activity, and immune response were investigated. Earthworm powder enriched with 1.0 mg/kg Se significantly increased the content of serum protein, albumin, and globulin and decreased the levels of serum glucose and triglycerides. Furthermore, SEP appeared to significantly increase the levels of GSH-Px, SOD, and catalase; decrease the level of nitric oxide; and enhance the immune response, including levels of IgG, IL-2, and interferon gamma.

ACKNOWLEDGMENTS

The present study was supported by the National Natural Science Foundation of China (No.41471410), and the Key Beijing Discipline of Ecology (XK10019440). This study received no specific grant from any funding agency, commercial or not-for-profit sectors.

Conflict of Interest Statement: The authors declare that there is no conflict of interest.

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