Shudi Erzi San relieves ovary aging in laying hens

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ABSTRACT Poultry meat and eggs are a primary source of animal protein. To meet the market needs, high yield laying hens are reared continuously, resulting in quick ovary aging. Thus, we investigated the anti-aging effects of Shudi Erzi San (**SES**) on laying hens. Sixty 300-day-old laying hens were divided into 2 experimental groups and a control group. The control group was fed on a basic diet, which was supplemented with 1% and 2% SES for experimental groups I and II, respectively. Egg quality and changes in serum hormones and blood-biochemical indicators of laying hens were determined. The rate of egg production was significantly higher in group Ⅱ than in both the control and group Ⅰ by 9.29 and 8.22 percentage points, respectively ($P < 0.05$). Eggshell strength of groups Ⅰ and Ⅱ were significantly higher than that of the control group ($P < 0.01$). Albumen height and Haugh Units of group Ⅱ were significantly higher than those of the control ($P < 0.05$). Serum levels of follicle stimulating hormone and estradiol in group Ⅱ were significantly higher than those of both the control and group Ⅰ ($P < 0.05$), whereas groups Ⅰ and Ⅱ had significantly higher serum levels of luteinizing hormone than the control ($P < 0.05$). Levels of superoxide dismutase (**SOD**) did not significantly differ between the control and group Ⅰ ($P > 0.05$), but SOD and malondialdehyde (**MDA**) levels in group Ⅱ were significantly higher and lower, respectively ($P < 0.05$) when compared to the control. Compared with the control, uric acid levels in groups Ⅰ and Ⅱ were significantly lower ($P < 0.05$), as was urea nitrogen in group Ⅱ ($P < 0.05$). Transcriptome and KEGG pathway analysis of ovarian tissues of laying hens showed a significant immune related signal pathway as the possible main regulator of a lysosome related signal pathway. Thus, supplementing chicken feed with SES improves egg production and quality and alleviates ovarian decline in laying hens.

Key words: Shudi Erzi San, ovary, aging, antioxidants, laying hens

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INTRODUCTION

Farmers use antibiotics to maintain animal health and improve animal production, yet the widespread use in animal production threatens food safety due to the exacerbating problem of drug residues. As per the 2020 announcement No. 194 of the Ministry of Agriculture and Rural Affairs of the People's Republic of China, the addition of antibiotics to feed was completely prohibited in China in order to reduce the harmful effects of antibiotics misuse and maintain food and public health safety. As the addition of antibiotics to feed is now prohibited, we need to adjust the nutritional design of feed, further improve animal nutrition and health care, and select new health products to replace antibiotics. High yield laying hens are more susceptible to ovarian oxidative stress and aging due to quick daily ovulations. At the fourth week after the laying begins, the laying rate of these hens is 50%, which peaks in the following 3 to 4 week. In this peak period, egg production reaches 90% to 97% and thereafter significantly decreases, seriously shortening the service life of laying hens and reducing their commercial value (Liu et al., 2018). The main performance problem of ovarian aging is that the number and quality of ovarian oocytes gradually decrease with increasing age (Younis, 2012). Normal reactive oxygen species (**ROS**) levels can promote follicle maturation and ovulation. However, with aging, ROS clearance efficiency in follicular fluid is significantly reduced, and antioxidant enzymatic activity gradually reduces. Excessively high levels of ROS cause loss of oocytes and granule cells in the follicles (Perheentupa and Huhtaniemi, 2009; Shkolnik et al., 2011; Ben-Meir et al., 2015). Most of the many

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factors that affect follicular development are activated by changes in the activity of the hypothalamus pituitary gonad axes (**HPG**). In general, gonadotropin-releasing hormone (**GnRH**) secreted by the hypothalamus, directly affects the pituitary gland. Antibiotics are predominantly used in clinical practice, yet antibiotic use is common in both livestock and crop agriculture as it increases yield. The flip side is that overuse of these antibiotics for the destruction of susceptible bacteria leads to emergence of antibiotic-resistant strains, which are then transferred to humans via the food chain (Duan et al., 2020). Accordingly, use of antibiotics is strictly forbidden during the laying period of laying hens.

Traditional Chinese medicines contain essential nutrients such amino acids, vitamins, alkaloids, and minerals. These components enable traditional Chinese medicines to improve the body's immunity, alleviate the body's immune stress, and improve production in animals. Shudi Erzi San (**SES**) is composed of *Lycium barbarum* L.—also known as the wolfberry—, *Cuscuta chinensis* Lam, and *Rehmannia glutinosa* (**RG**). It can contribute to anti-aging as it scavenges free radicals, improving the level of anti-oxidants and immune regulation. In traditional Chinese Veterinary Medicine, Wolfberry and *Cuscuta chinensis* Lam have the function of tonifying liver and kidney, and RG has the function of nourishing blood. Wolfberries were first used as medicinal plants about 2,300 years ago (Li, 2007). Although wolfberries are increasingly popular due to their health benefits, many have forgotten their anti-aging properties (Chang and So, 2008). *Lycium barbarum* polysaccharides (**LBP**) increase level of hormones, antioxidant enzymes and antioxidants, scavenge free radicals, and reduce lipid peroxide production (Luo et al., 2006; Cheng and Kong, 2011; Xiao et al., 2013; Wang et al., 2014; Meng et al., 2015). *L. barbarum* has nutrients such as LBPs, vitamins and carotenoids that have antioxidant, anti-inflammatory, anti-aging, anti-diabetes, liver protection, and immunomodulatory properties. *L. barbarum* is often clinically used to enhance reproductive functions (Zhang et al., 2015). *Cuscuta chinensis* Lam is a member of Convolvulaceae family. It has antioxidant properties and largely contributes to the reproductive system (Qin et al., 2019) and is thus regarded as a nutritional supplement to promote human health and prevent oxidation-related diseases (Yang et al., 2009). *C. chinensis* Lam reduced levels of ROS and malondialdehyde (**MDA**), and significantly reduced apoptosis of germ cells (Yen et al., 2008). *R. glutinosa* is an antioxidant, and has been used as one for millennia (Zhang et al., 2004). The addition of *R. glutinosa* officinalis to the diet of mice had an anti-aging effect as it reduced ROS levels (Bai, et al., 2018). In summary, decline in ovarian functions of laying hens causes a dramatic decline in egg production. In this study, SES was used to alleviate ovarian aging in laying hens and thus delay ovarian decline, which ultimately increased egg production.

MATERIALS AND METHODS

Drugs and Reagents

Wolfberries, *C. chinensis* Lam and *R. glutinosa* were purchased from the Chinese Herbal Medicine Market, Bozhou, Anhui Province, China. After removing impurities, they were dried and ground through a 100 mesh sieve. SES was formed by mixing the three ingredients in a ratio of 1:1:1—this ratio is based on ensuring ideal biological activity, compatibility, and pharmacological action properties. Kits that tested serum levels of cholinesterase (**CHE**), alkaline phosphatase (**AKP**), total protein (**TP**), aspartate aminotransferase (**AST**), alanine aminotransferase (**ALT**), blood glucose (**GLU**), urea nitrogen (**UREA**), creatinine (**CRE**), and uric acid (**UA**) were purchased from Nanjing Jiancheng Biotechnology Research Institute, Nanjing, Jiangsu, China. Superoxide dismutase (**SOD**), malondialdehyde (**MDA**), and catalase (**CAT**) were similarly purchased from Nanjing Jiancheng Biotechnology Research Institute.

Animal Grouping and Treatment

Experiments were conducted at animal laboratories of Hebei Agricultural University, following approval of study protocols by the Animal Protection and Utilization Committee of the Hebei Agricultural University. Laying hens were kept in clean and well-ventilated chicken houses, in a total of 20 cages that each contained 3 hens. Each cage had a water kettle and food picker and was maintained in good hygiene conditions. Eggs were collected every afternoon. Sixty healthy 300-day-old laying hens were randomly divided into control group, 1.0% SES group (group Ⅰ) and 2.0% SES group (group Ⅱ), and fed on three treatments —basal diet, basal diet supplemented with 1.0% SES and basal diet supplemented with 2.0% SES, and the feeding period was 4 weeks. All diets were formulated to meet nutrient requirements (Table 1). Blood samples (5 mL) were collected from wing veins of laying hens on day 28. Samples were retained at room temperature for 30 min, then

Table 1. Basal diet.

Ingredients	Contents (%)
Corn	64
Soybean meal	25
Limestone	8.3
Salt	0.3
Dicalcium phosphate	1.8
Vitamin premix[1]	0.3
Mineral premix[2]	0.02
L-Lysine HCl	0.01
DL-Methionine	0.27
Total	100

[1]Vitamin premix supplied (per kg of diet): Vitamin A, 6,000 IU; Vitamin D3, 1,500 IU; Vitamin K3, 4.2 mg; Vitamin B1, 3 mg; Vitamin B2, 10.2 mg.

[2]Mineral premix provided (per kg of diet): Cu ($CuSO_4 \cdot 5H_2O$), 6.8 mg; Fe ($FeSO_4 \cdot 7H_2O$), 66th mg; Zn ($ZnSO_4 \cdot 7H_2O$), 83 mg; Mn ($MnSO_4 \cdot H_2O$), 80 mg.

centrifuged at 3,000 rpm/min for 10 min. Serum was stored in 1.5 mL tubes at −20°C. Ovaries from laying hens were stored at −80°C.

Production Performance and Egg Quality

During the test, the number of eggs was recorded repeatedly every day to calculate the egg laying rate. On the 28th day of the experiment, 10 eggs were randomly selected from each replication. To calculate the egg shape index, vertical and horizontal axes of eggs were measured with vernier calipers. An EMT-5200 egg quality analyzer (Robotmation, Kyoto, Japan) was used to measure egg weight, protein height, Haugh Unit, and egg yolk color. The eggshell strength and thickness were measured by the eggshell strength and thickness testers, respectively. All egg quality tests were carried out at the Hebei Institute of Animal Husbandry and Veterinary Medicine.

Serum Traits Measurements

Blood serum samples were analyzed for CHE, AKP, TP, AST, ALT, GLU, UREA, CRE, and UA. CHE was determined by colorimetry, AKP by microplate method, TP by Coomassie brilliant blue method, AST, ALT, and CRE by microplate method, GLU by colorimetry and UREA by urease method.

Biochemical Measurements

Indexes of oxidative stress—SOD, CAT, and MDA—were measured in serum samples, using analytical kits (Nanjing Jiancheng Biological Company, Nanjing, Jiangsu, China), according to manufacturer's instructions.

Determination of Hormone Indicators in Serum

The serum levels of FSH, LH and estradiol were measured by radioimmunoassays—FSH was mainly detected by non-equilibrium radioimmunoassays. Serum was separated from venous blood and mixed overnight with reagents at 4°C using a separator, then placed at room temperature for 15 min before centrifugation for 15 min. The radioactivity count (CPM) of each tube's precipitation was measured via a radioimmunoassay. LH levels were mainly determined by the advanced PR method with highly specific antibodies. All assays were conducted at Beijing Leibotieri Technology Co. Ltd.

Analysis of mRNA Transcription Levels in Ovaries of Layer Hens

Gene ontology (http://geneontology.org/)—a standardized gene function classification system—provides a set of dynamically updated yet regulated descriptions of attributes of genes and their products. GO covers three aspects: molecular function, cellular component, and biological process. During the analysis, we used differentially expressed genes that had GO annotations to calculate the gene list and number of each term and then calculate *P*-values by hypergeometric distribution methods (at an alpha level of 0.05). This was used to identify GO annotations that were significantly enriched in differentially expressed genes compared to background expression levels to determine their main biological functions. Kyoto Encyclopedia of Genes and Genomes (**KEGG**) (http://www.kegg.jp/) is a database integrating genomic, chemical, and system functional information. We used its cluster profiler for KEGG enrichment analysis. During the analysis, we used differentially expressed genes annotated by KEGG pathways to calculate the gene list and number of each pathway and then calculated the *P*-value through hypergeometric distribution methods (at an alpha level of 0.05). To determine the main biological functions of differentially expressed genes, these *P*-values were used to identify KEGG pathways that were enriched with differentially expressed genes compared to background expression levels of the whole genome. The differentially expressed genes were first identified from analysis of sequenced reads and annotated in GO and KEGG databases before verification via reverse transcription quantitative polymerase chain reactions (**RT-PCR**). The sequence is shown in Table 2.

Table 2. Primer sequences.

Target genes	GenBank accession No.	Primers sequences	Product length/bp
CAT	NM_001031215.2	F:AGCCGCATGTCCGTTTCAG R:ACACAGCCTTTGGCGTTCATC	120
SOD1	NM_205064.1	F:TCTTACCGGACCACACTGCATC R:ACGAGGTCCAGCATTTCCAGTTA	115
SOD2	NM_204211.1	F:GATAGCAGCCTGTGCAAATCAAGA R:GCATGTTCCCATACATCGATTCC	84
Mgst1	XM_015290135.2	F:CAGATGTTGAACGTGTACGCAGAG R:TGGACAGATCAGGGCCACAG	105
Prdx3	XM_426543.5	F:GCATAACAGGAGCGTTAACAAGCA R:GAGGCAGATTCAAGCAGGTAAACA	145
Actb	NM_205518.1	F:ATTGTCCACCGCAAATGCTTC R:AAATAAAGCCATGCCAATCTCGTC	113

Statistical Analysis

Sequenced reads were analyzed with a DESeq2 software package to identify differentially expressed genes using the following inclusion criteria: $\log^2$ (foldchange) > 1 & P-value ≤ 0.05. The clusterProfile software was used to analyze the GO and KEGG function enrichment of differentially expressed genes. An alpha level of 0.05 was used.

Statistical analysis was done in SPSS 19.0 (IBM corporation, Armonk, NY). All data were presented as mean ± standard deviation. One-way analysis of variance (**ANOVA**) followed by Tukey's test was used for multiple comparisons, and those with $^{*}P < 0.05$ or $^{**}P < 0.01$ were considered statistically significant.

RESULTS

Effect of SES on the Production Performance Index

The rate of egg production was significantly higher ($P < 0.05$) in groupⅡ than both in the control and groupⅠby 9.29% ($P < 0.05$) and 8.22% ($P < 0.05$), respectively (Table 3). The egg shape index and weight of groupsⅠandⅡdid not significantly differ from the control ($P > 0.05$). The eggshell strength of groupsⅠandⅡwere significantly higher than the control ($P < 0.01$). The albumen height and Haugh units were both significantly higher in groupⅡthan of the control group ($P < 0.05$). Yolk color of groupsⅠandⅡwere significantly higher than the control ($P < 0.05$). There were no significant differences in eggshell thickness among the three groups ($P > 0.05$).

Effect of SES on Antioxidant Indexes

Levels of SOD in group Ⅰ were not significantly different ($P > 0.05$) from the control, whereas those of group Ⅱ were significantly higher ($P < 0.05$; Figure 1). Levels of CAT did not significantly differ among all three groups ($P > 0.05$). MDA levels in group Ⅱ were significantly lower than those of the control ($P < 0.05$).

Effect of SES on Reproductive Hormones in Laying Hens

Serum levels of FSH and estrogen were significantly higher in groupⅡ than those of both groupⅠand the control ($P < 0.05$). LH levels in groupsⅠandⅡwere significantly higher than in control ($P < 0.05$; Figure 2).

Effect of SES on the Biochemical Indexes

GroupsⅠandⅡdid not significantly differ from the control in AKP, CHE, and TP ($P > 0.05$; Table 4) as well as in ALT, AST, and GLU ($P > 0.05$). However, GroupsⅠandⅡhad significantly lower UA than the control ($P < 0.05$). GroupⅡhad significantly lower UREA than the control ($P < 0.05$) but did not have significantly different CRE levels ($P > 0.05$).

Effect of SES on Immunoglobulin

Immunoglobulins IgA, IgG, and IgM are related to immunity (Figure 3). IgA levels in group I and group II increased significantly compared to the control group ($P < 0.05$), but there were no significant differences in levels of IgG and IgM ($P > 0.05$).

Transcriptome Analysis

The comparative analysis of gene expression levels between samples is an important index for assessing the reliability of experimental designs and rationales of sample selection (Figure 4A). Before differential expression analysis, the correlation of gene expression levels between samples should be checked. Thus, we used Pearson's correlation coefficient to measure the correlation of gene expression levels between samples. The closer the correlation coefficient is to 1, the higher the similarity of expression patterns between samples—0.8 to 1 indicates a very strong correlation coefficient. In this study, the difference in correlation coefficient between the 2 groups was greater than 0.8, indicating that the sampling and experimental results were reliable. The volcanic map shows gene distribution, differences in gene expression and significant results (Figure 4B). The abscissa is the $\log^2$ fold change, and the ordinate is the significance level of the control and treatment groups. When compared to the control, there were 185 differentially expressed genes (**DEGs**) in the SES group, of which 134 genes were downregulated and 51 genes were upregulated (Figure 4C). We use the phatmap software package in R to conduct a two-way cluster analysis on the union of different genes and samples in all

Table 3. Effect on egg quality.

Groups	Control group	Ⅰ Group	Ⅱ Group
Laying rate /%	77.14 ± 6.26	78.21 ± 3.38	86.43 ± 6.24*
Egg shell strength	24.11 ± 6.68	36.29 ± 4.92**	38.28 ± 5.04**
Egg weight	61.79 ± 1.84	61.49 ± 3.38	58.23 ± 3.05
Albumen height /mm	5.49 ± 0.65	5.43 ± 0.98	6.28 ± 0.77*
Yolk color	7.13 ± 0.58	8.13 ± 0.22**	8.36 ± 0.24**
Haugh unit	71.95 ± 5.97	74.16 ± 7.85	78.35 ± 5.79*
Egg shell thickness /mm	0.35 ± 0.03	0.35 ± 0.03	0.37 ± 0.01

Control group (basal diet), I group (containing 1.0%SES) and II group (containing 2.0% SES).
*$P < 0.05$, compared with the control group.
**$P < 0.01$, compared with the control group.

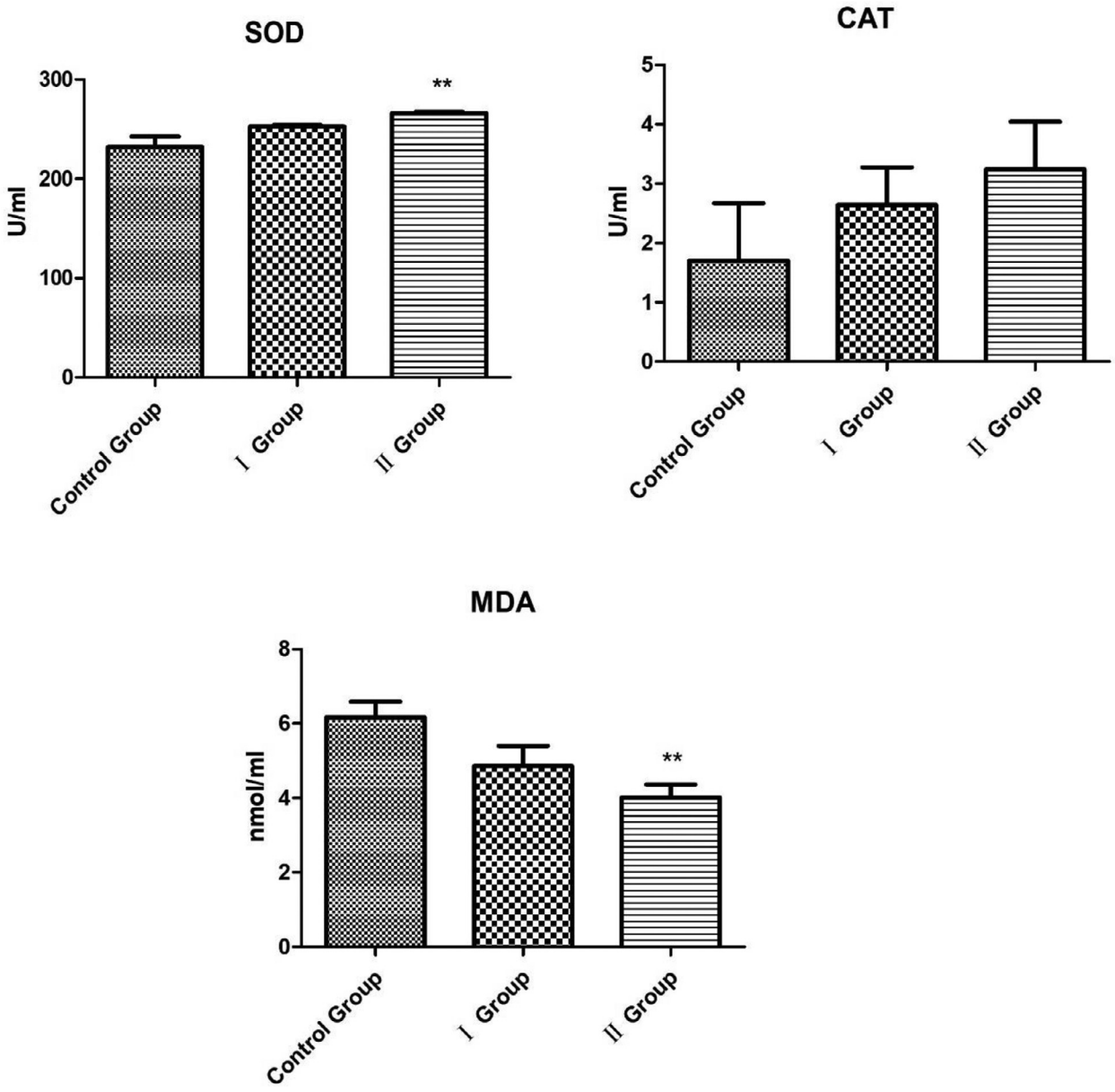

Figure 1. Effect of SES on antioxidant indexes. The data were expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviation: Shudi Erzi San.

comparison groups. We clustered based on the expression level of the same gene in different samples and the expression pattern of different genes in the same sample. Euclidean methods were used to calculate distances and a hierarchical clustering and complete linkage method was used for clustering. Through cluster analysis, we classified those with high expression correlations among samples into one category to further analyze correlations among them (Figure 4D). The 20 most significant results were selected from the GO enrichment analysis results and represented in a columnar chart, in which the abscissa was the number of DEGs annotated with GO terms/the total number of genes annotated to a specific GO term, and the ordinate was the GO term. Most significantly enriched DEGs were annotated as regulators of immune response (Figure 4E). DEGs were analyzed with KEGG pathways, and the 20 most significant pathways were selected from KEGG enrichment results and used to plot a scatter diagram. The abscissa was the ratio of the number of DEGs to the total number of DEGs, and the ordinate was the KEGG pathway. A color range of red to blue was used to indicate the significance of enrichment. Immune related lysosomal pathways were significantly enriched (Figure 4F). Expression levels of *SOD1* and *Prdx3* genes were significantly higher in group Ⅱ than in the control ($P < 0.05$). Compared to the control, expression levels of *CTSS, acp5*, and *atp6v0d2* genes in group II were significantly lower ($P < 0.05$). Expression levels of *SOD2, CAT*, and *Mgst1* genes did not significantly differ among the three groups ($P > 0.05$; Figure 5).

DISCUSSION

Traditional Chinese medicine is now globally recognized and widely used. In diets of laying hens, the addition of Chinese herbal mixtures (**CHM**) had no

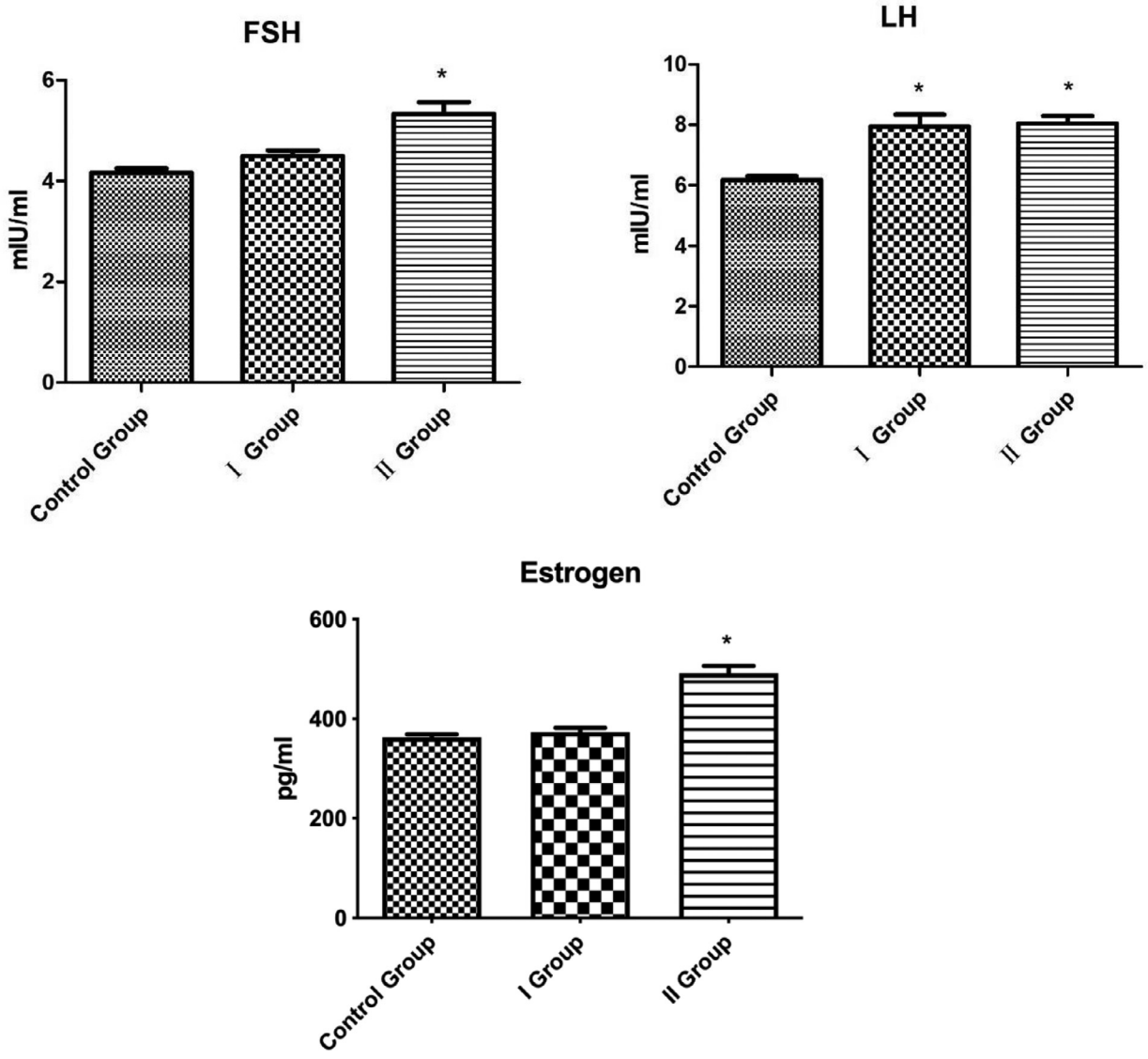

Figure 2. Effect of SES on reproductive hormones in laying hens. The data were expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviation: Shudi Erzi San.

significant effect on egg shape index and eggshell thickness (Li et al., 2016). The most important criterion for a consumer's choice of eggs is the yolk color. Egg yolk color is mainly determined by carotenoids and other pigmented substances —laying hens do not biosynthesize them— in the feed (Macit et al., 2009). Lutein in carotenoids is absorbed by laying hens and deposited in eggs. The wolfberry component of SES has strong antioxidant activity due to functional components such as LBP, flavonoids, carotenoids, vitamins, and betaine. Wolfberries have an extremely high content of carotenoids, which are ultimately deposited in egg yolks, improving their color to one that is more welcoming to consumers — (Ketta and Tůmová, 2016). Relatedly, supplementing feed with CHM significantly improved the egg yolk color (Li et al., 2016). In this study, supplementing diets with SES had no significant effect on egg shape index, egg weight, and eggshell thickness. However, supplementing diets with 2% SES significantly improved the Haugh unit of eggs — Haugh unit is the best index for measuring protein quality (Zhao et al., 2015). High and medium quality eggs have Haugh units higher than 72 and between 60 and 72, respectively (USDA, 2000). Although all three groups in this study produced high quality eggs of >72 Haugh units, SES supplementation further improved their Haugh units. Eggshell strength is an important index of egg quality and is very important for egg transportation. Our results showed that hens fed on diets supplemented with SES had higher egg laying rates than those fed on basic diets. Supplementing feed with traditional Chinese medicine improved the feeding efficiency of laying hens (Li et al., 2016). This may be because it improved the taste and palatability of poultry feed as well as digestion and absorption of nutrients in the digestive tract (Wenk, 2003; Almuhanna et al., 2011). Moreover, the increased laying rate may result from active components of traditional Chinese medicine. Traditional Chinese medicine is rich in protein, carbohydrates, vitamins and minerals, which all play important roles in maintaining nutritional balance and improving growth performance (Li et al., 2016). Wang and colleagues showed that supplementing diets with

Table 4. Effect on the biochemical indexes of layers.

Groups	Control group	Ⅰ Group	Ⅱ Group
CHE (U/L)	1,062.19 ± 92.14	1,096.27 ± 82.07	1,084.73 ± 95.24
AKP (King unit/100 mL)	35.81 ±4.63	32.94 ± 6.39	33.05 ± 7.95
AST(U/L)	1,551.96 ± 75.74	1,543.02 ± 70.16	1,568.65 ± 71.43
ALT(U/L)	13.41 ± 1.24	13.05 ± 1.75	12.67 ± 2.26
GLU (mmol/L)	6.69 ± 1.70	7.40 ± 0.72	7.10 ± 0.61
TP (g/L)	141.24 ± 23.96	130.22 ± 21.51	144.18 ± 43.42
UREA (mmol/L)	1.03 ± 0.09	0.92 ± 0.13	0.86 ± 0.11*
CRE (μmol/L)	31.78 ± 8.67	26.28 ± 8.59	23.28 ± 4.11
UA (μmol/L)	268.32 ± 12.40	244.14 ± 11.60*	230.80 ± 14.55*

*$P < 0.05$, compared with the control group.

suspensions of the Chinese herb *Forsythia* improved production performance in birds (Wang et al., 2008). Ma and colleagues report that supplementing diet with traditional Chinese medicine (*L. lucidum* and *Schisandra chinensis*) significantly improved the laying rate of layers, corroborating our findings (Ma et al., 2005).

Serum biochemical indicators reflect animal health and are important markers of animal nutrition and physiology (Alagawany and El-Hack, 2015). They also show relationships between the internal environment of organisms and their nutrition, especially the relationship between serum immune and antioxidant indexes. In this

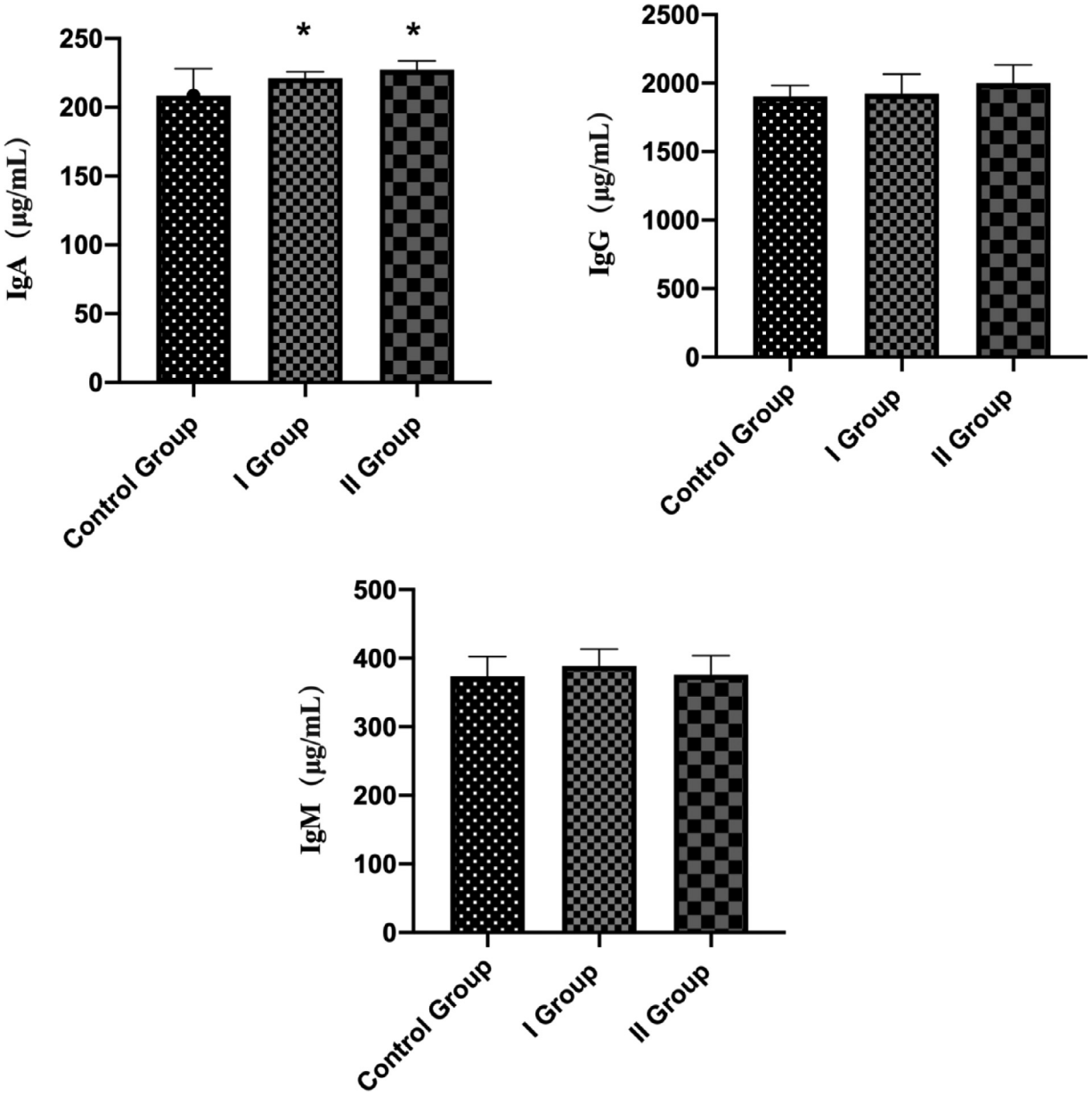

Figure 3. Effect of SES on immunoglobulin. The data were expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$. Abbreviation: Shudi Erzi San.

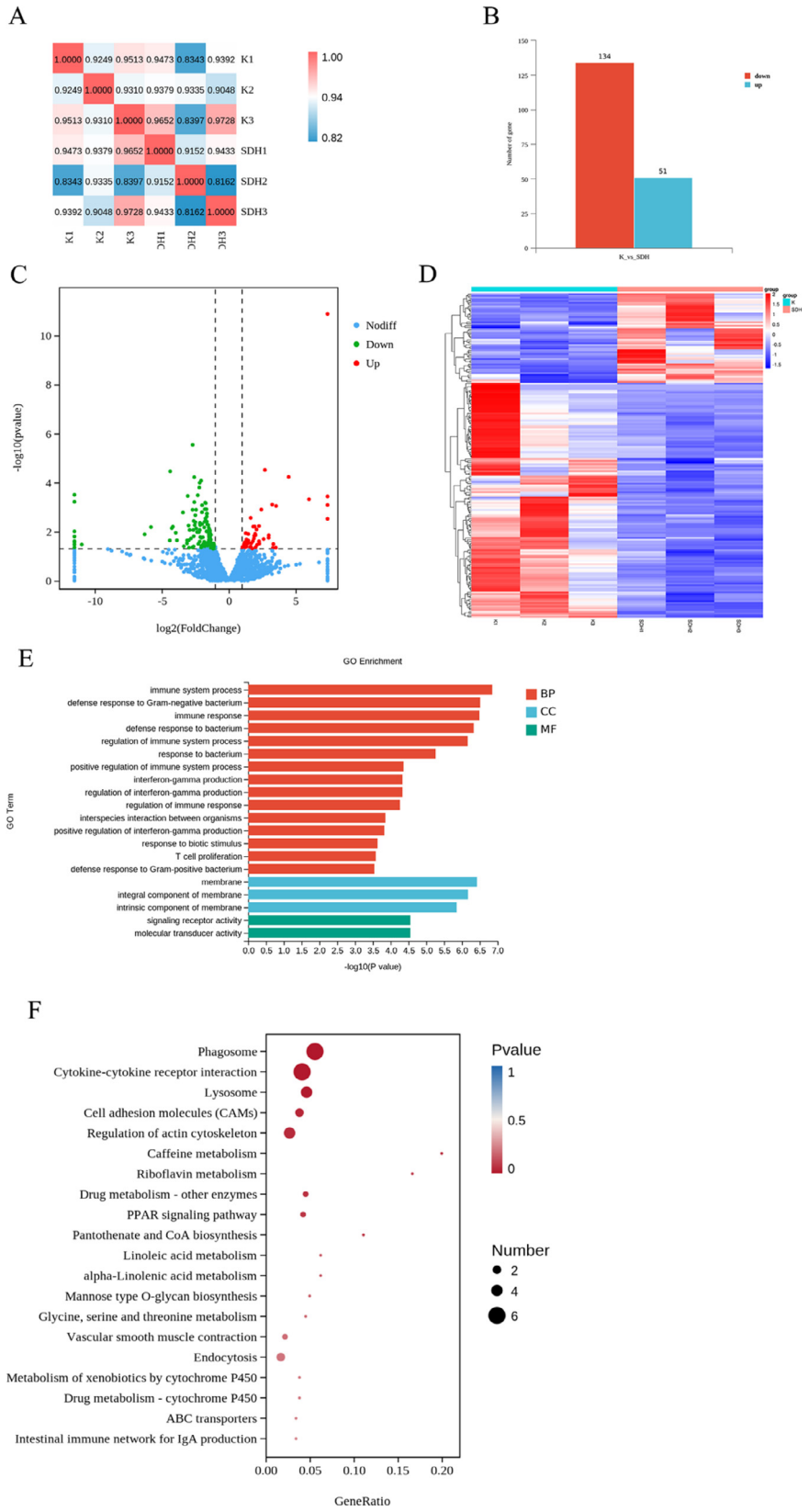

Figure 4. Transcriptome sequencing analysis. (A) Intersample correlation. (B) Differential gene expression. (C) Significant differentially expressed genes. (D) Cluster analysis. (E) Enrichment of GO pathway. (F) Enrichment of KEGG pathway.

study, supplementing diets with SES had no significant effect on CHE, AKP, AST, ALT, GLU, TP, and CRE levels but reduced UREA and UA levels. Relatedly, SES supplementation had no significant effect on serum AST and ALT activity. In poultry, serum AST and ALT activities are largely associated with age, as they gradually decrease after adulthood (Króliczewska et al., 2008). Valizade and colleagues showed that supplementation with high amounts of threonine had no significant effect on the serum AST levels in male broilers (Valizade et al., 2016). TP and UA reflect the metabolic state of proteins in the body, which is closely related to the

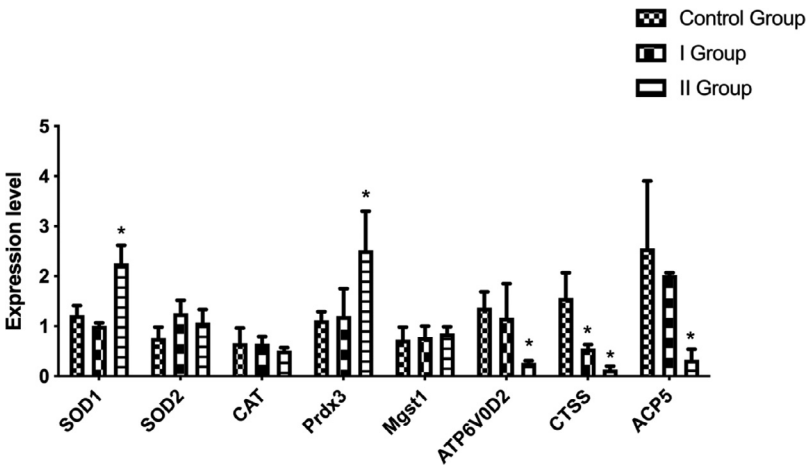

Figure 5. Relative expression levels of SOD1, SOD2, CAT, Prdx3, Mgst1, CTSS, ACP5, ATP6V0D2 genes. $^{*}P < 0.05$, $^{**}P < 0.01$.

activity of lysozyme and level of immunity. UA and UREA are the main indicators of kidney function. UA is the final product of amino acid metabolism in birds, and thus reflects their metabolism and immune function. If kidney function is impaired, excretion of UA is blocked, resulting in increased UA serum levels.

Antioxidant enzymes in follicular fluid are closely related to follicular maturation, and either high levels of oxidation products or low antioxidant capacities of oocytes disrupt the redox balance and lead to oxidative stress (Mayer et al., 2002). ROS are harmful products of aerobic metabolism, but when within their normal range, they regulate the activation of transcription factors (Finkel, 2011). The imbalance of ROS causes oxidative stress leading to peroxidation of macromolecules, such as proteins and nucleic acids, which affects health and production performance. High levels of ROS directly damage macromolecules. Under physiological conditions, ROS generation is controlled by endogenous antioxidants, such as SOD and CAT (Loh et al., 2006; Sugawara and Chan, 2003). CAT is an important antioxidant enzyme that largely contributes to eradicating free oxygen radicals and lipid peroxidation (Cheng and Kong, 2011). However, increasing age results in decreasing levels of SOD and CAT, which in turn increases levels and accumulation of free oxygen radicals, resulting in the oxidation of unsaturated fatty acids, which ultimately produces many lipid peroxides. Lipid peroxidation eventually produces MDA, and this is used to indirectly measure a body's ability to scavenge free radicals and its antioxidant capacity (Blumberg, 2004; Szeto, 2006). LBP increases antioxidant enzyme activity and decreases MDA content in a dose-dependent manner (Yang et al., 2017). Zhang and colleagues showed that LBP significantly improved antioxidant capacities, as the level of SOD increased in a dose-dependent manner whereas levels of MDA significantly decreased (Zhang et al., 2014). After ovulation, oxidative stress occurs, with the resultant free oxygen radicals destroying biofilms, nucleic acids, reducing the developmental ability of oocytes and further inhibiting immune functions (Miyamoto et al., 2010). Our study showed that SES significantly increased levels of SOD and reduced levels of MDA. Thus, SES is an antioxidant that alleviated the oxidative stress in ovaries of laying hens.

With increasing age, functions of the immune system gradually decrease. IgA, IgG, and IgM are important indicators for evaluating the immune status of poultry. They confer resistance to pathogen invasion and enhance immune regulation (Yang et al., 2012). IgA is the main component of the mucosal defense system and is an important barrier in the respiratory mucosa. IgG is one of the active components of humoral immune responses to infection and thus participates in antibacterial and antiparasitic reaction processes (Vaezirad et al., 2018). Our findings show that SES significantly increased serum IgA levels. Based on these increases in serum globulin levels, we hypothesize that SES has a beneficial effect on the immunity of laying hens. Wolfberry contains a large number of carotenoids, which can regulate immune response by increasing antibodies (Rajput et al., 2013). Relatedly, research showed that *C. chinensis* Lam significantly enhanced immune function in mice. Reproductive organ and follicular development, maturation and ovulation in layers are mainly regulated by the hypothalamic-pituitary-gonadal axis; this is thus through the secretion of hormones (Akazome et al., 2002). FSH, LH, and estradiol are important indicators for evaluating the reproductive system. Laying hens produce eggs through synergies of secreted hormones. FSH and LH are glycoprotein hormones that are the main regulators of poultry follicular development and promote the secretion of other hormones such as estradiol (Lewis et al., 2005). FSH stimulates granulosa cell maturation and contributes to follicular development and ovulation. Secretions from the anterior pituitary gland promote follicular growth and maturity, which causes ovulation (Johnson, 1993). LH induces follicle growth and triggers ovulation. Levels of serum FSH and E2 in group Ⅱ were significantly higher than in both the control and groupⅠ. LBP alleviated oxidative stress and hormone secretion disorder induced by diethylstilbestrol (**DES**) in adult male hamsters (Zhang et al., 2014). *C. chinensis* extracts increased levels of sex hormones in rats (FSH and E2 levels increased; Zhang, et al., 2019), which is consistent with our results. Our findings suggest

that SES promotes hormone secretion and may be significant in maintenance of laying hens. To explore the possible mechanisms by which SES improves ovaries of laying hens, we sequenced transcriptomes of ovaries. We found 185 DEGs, of which 134 were downregulated and 51 were upregulated. Most DEGs were associated with immunity. KEGG pathway analysis showed that the immune related lysosomal pathway was significantly enriched. Cells transport aged and damaged proteins or organelles into lysosomes degrade them to maintain the stability of the internal environment of cells, in a process called autophagy. As aging is closely related to autophagy, with increasing age, autophagy capacities decrease, resulting in accumulation of harmful proteins (Levine et al., 2011; Madeo et al., 2011). We verified DEGs, and the results corroborated those from transcriptome analysis.

In summary, SES reduces the oxidative stress caused by ovarian age. SES possibly regulated immune related pathways and may have contributed to cellular homeostasis via regulation lysosomes. Thus, SES alleviated aging in the ovaries of laying hens.

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DISCLOSURES

The authors have no conflicts of interest.

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