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Biological Trace Element Research

ISSN 0163-4984

Biol Trace Elem Res
DOI 10.1007/s12011-016-0646-y

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Effects of Dietary Selenium Supplementation on Seminiferous Tubules and SelW, GPx4, LHCGR, and ACE Expression in Chicken Testis

Ahmed Khalid[1,2] · **Nagam Khudhair**[3,4] · **Huang He**[1] · **Zheng Peng**[1] · **Tian Yaguang**[1] · **Zhang Guixue**[1]

Received: 15 January 2016 / Accepted: 9 February 2016
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Abstract We investigated the effects of dietary selenium (Se) supplementation on the development of chicken testis and the expression of selenoprotein W (SelW), glutathione peroxidase4 (GPx4), luteinizing hormone/choriogonadotropin receptor (LHCGR), and angiotensin converting enzyme (ACE). Sixty roosters were assigned randomly into the control group fed with a basic diet (containing 0.3 mg Se/kg) and the experimental group fed with a diet (containing 0.6 mg Se/kg). The testes were collected individually at age of 6, 9, and 12 weeks. Se was supplemented in chicken feed for 15 days before sampling. The results indicated that dietary Se affected the number of cells in the seminiferous tubules and viability of Sertoli cells in vitro culture. SelW and GPx4 expression in the testes increased significantly in the experimental group compared to that in the control group. LHCGR expression in the testes increased significantly in the experimental group after 12 weeks compared to that in the control group. In contrast, ACE expression was inhibited in the experimental group compared to that in the control group. These results suggest that dietary supplementation with Se improved development of the seminiferous tubules at the cellular level and that SelW, GPx4, LHCGR, and ACE are involved.

Keywords Selenium · Chicken · Male reproduction · Sertoli cell · Gene

✉ Zhang Guixue
gxzhang@neau.edu.cn

[1] College of Animal Science and Technology, Northeast Agricultural University, Harbin 150030, China

[2] Department of Animal Production, College of Agriculture, University of Tikrit, Tikrit, Iraq

[3] Key laboratory of Dairy Science of Education Ministry, Northeast Agricultural University, Harbin 150030, China

[4] Biology Department, Education College for Women, Al-Anbar University, Ramadi 31001, Iraq

Introduction

Dietary deficiencies of several nutrients, such as vitamins and trace minerals, have been associated with decreased fertility in animals [1]. The trace mineral selenium (Se) is a known component of spermatozoa and is required for spermatogenesis [2]. Se is an essential trace mineral nutrient that has been linked to various physiological functions. Se helps protect against oxidative damage of sperm, which is crucial for male fertility; therefore, Se plays an important role in the maintenance of male fertility [3]. Dietary Se level regulates the expression of selenoproteins (Sels) [4]. At least 25 Sels have been identified in animals and humans and chickens have at least 23 Sels [5]. The glutathione peroxidase family (GPx) was the first Sel family identified with known function [6]. Some studies have reported that selenoprotein W (SelW) and glutathione peroxidase4 (GPx4) protect cells against damage caused by reactive oxygen species (ROS) [7]. SelW plays an antioxidant role in mammals, and chicken SelW possesses an antioxidant function similar to that of mammalian homologs despite the lack of Cys37 in the peptide [8]. Se and GPx levels increase in animals treated with excess Se [9], and GPx4 mRNA levels are downregulated by excess Se in chickens [10]. Testicular Sel15 and thioredoxin reductase 1 mRNA levels are attenuated when dietary Se is increased from 0.3 to 3.0 mg Se/kg, whereas Gpx1 expression increases in the pig liver [11].

A testicular angiotensin-converting enzyme (ACE) isozyme likely plays important functional roles in male reproduction. The ACE gene encodes both a somatic isozyme found in blood and several other tissues, such as the epididymis and, a testis-specific ACE isozyme. ACE levels in sperm samples

Published online: 22 February 2016

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Table 1 List of gene-specific primers

Primer	Sequences	Product (bp)	Gene bank no.
SelW	5′CTCCGCGTCACCGTGCTC′3 5′CACCGTCACCTCGAACCATCCC′3	150	GQ919055
LHCGR	5′GCAACGAATCGCTGA′3 5′GTTGAAGAAATATCCAGG′3	178	NM_204936.1
GPx4	5′GCCACCTCCATCTACGACTTC′3 5′TCCTTCAGCCACTTCCACAG′3	335	AF498316
ACE	5′ACATCCCTGCCAACGTCCCCT′3 5′GTTCTCTGTGTTCTTCTTCA′3	293	NM_001167732.1
β-actin	5′CCGAGAGAGAAATTGTGCGTGAC′3 5′TCGGGGCACCTGAACCTCTC′3	166	L08165

GPx4 glutathione peroxidase 4, *ACE* angiotensin I converting enzyme, *LHCGR* luteinizing hormone/choriogonadotropin receptor, *SEPW1* selenoprotein W, 1

from normozoospermic men are lower than those in sperm from oligo asthenozoospermic men [12]. In addition, some evidence suggests that testicular weight decreases markedly in Mas-deficient mice [13]. Therefore, substantial evidence implies that ACE regulates spermatogenesis. The luteinizing hormone/choriogonadotropin receptor (LHCGR) mediates the actions of LH on Leydig cell steroidogenesis and testosterone production to support germ cell development and final differentiation of haploid spermatids into spermatozoa (spermatogenesis) [14]. Se presents in the pituitary gland with its normal rates [15], thus it is considered one of the basic elements affecting LH secretions of that gland. Concerning the mice lacking LH or LH receptor (LHCGR), adult Leydig cell numbers are about 10 % of normal and testosterone levels are essentially undetectable [16, 17]. The critical role of Se in the modulation of redox status of testicular cells also emphasized the importance of Se status for reproductive health [18]. The aim of this study was to evaluate the effects of Se dietary supplementation within the allowable nutritional level on the development of testicular tissue, Se1W, GPx4, ACE, and LHCGR gene expression in testes and Sertoli cell viability in vitro.

Material and Methods

Animals and Tissue Sampling

Sixty chickens were divided randomly into two groups and were housed in cages until the age of 12 weeks. The control group was fed a basal diet (basic diet containing 0.3 mg Se/kg) and the experiment group was fed the experimental diet (basic diet containing 0.3 mg + 0.3 mg (inorganic selenium) Se/kg). The photoperiod was 16 L/8D, and water was provided ad libitum. Ten roosters from each group were selected randomly after 6, 9, and 12 weeks of feeding and euthanized by argon

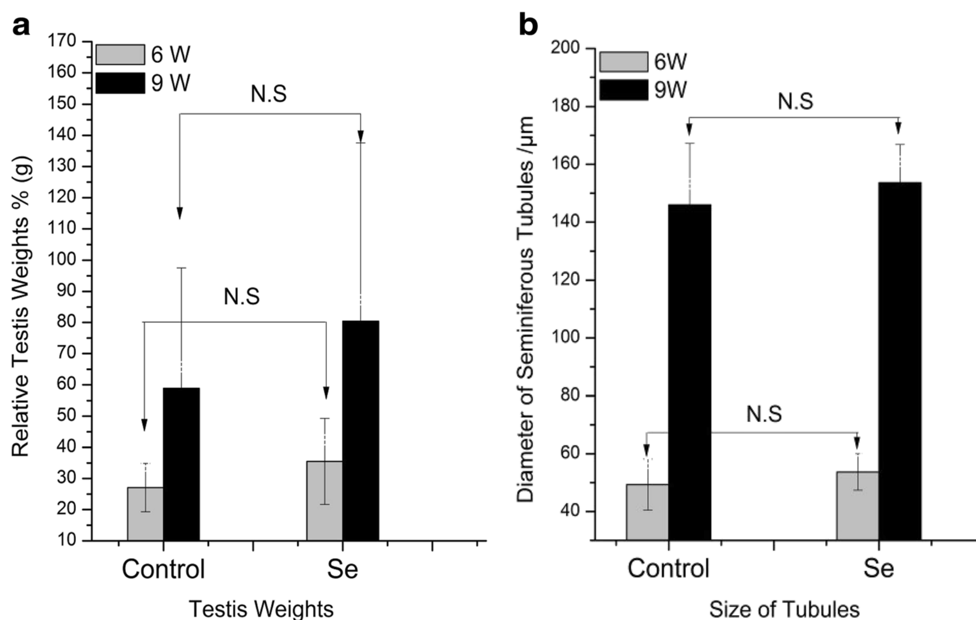

Fig. 1 Effects of selenium on testicular weight and seminiferous tubule size in 6- and 9-week-old chickens. **a** Testicular weight. **b** Seminiferous tubule size. ($P > 0.05$) ($n = 8$)

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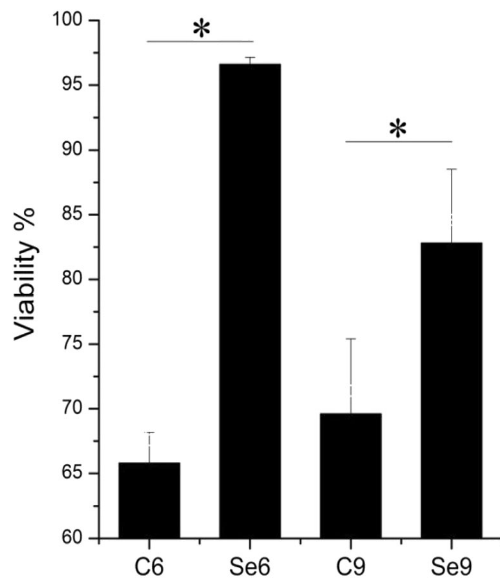

Fig. 2 Sertoli cell viability in 6- and 9-week-old chickens in the control and experimental groups. (C 6, 9) = Control at 6 and 9 weeks. (Se 6, 9) = Selenium experimental group at 6 and 9 weeks. ($P < 0.05$) ($n = 5$)

asphyxiation followed by cervical dislocation. The testes were removed quickly, weighed, and stored for further analyses. Half of the fresh testes were placed in a PBS solution, and Sertoli cells were prepared according to the method of Khalid et al. [19]. The other half of the testes were cut into two parts; half was prepared for histological observations, and the other half were frozen in liquid nitrogen and stored at −80 °C for real-time polymerase chain reaction (RT-PCR) analysis.

Measurement of Relative Testis Weight

Body and testicular weights of both groups were measured at the end of 6 and 9 weeks using an electronic balance (Mettler–Toledo, Columbus, OH, USA). Relative testicular weight was calculated by W = (testis weight (g) / body weight (g) × 100).

Sertoli Cell Viability and In Vitro Culture

Sertoli cells were harvested from the seminiferous tubules, [19] and viability was estimated before culture using the trypan blue dye test. First-passage cells were counted with the Invitrogen Countess™ Automated Cell Counter (Carlsbad, CA, USA) and photographed under a phase contrast microscope.

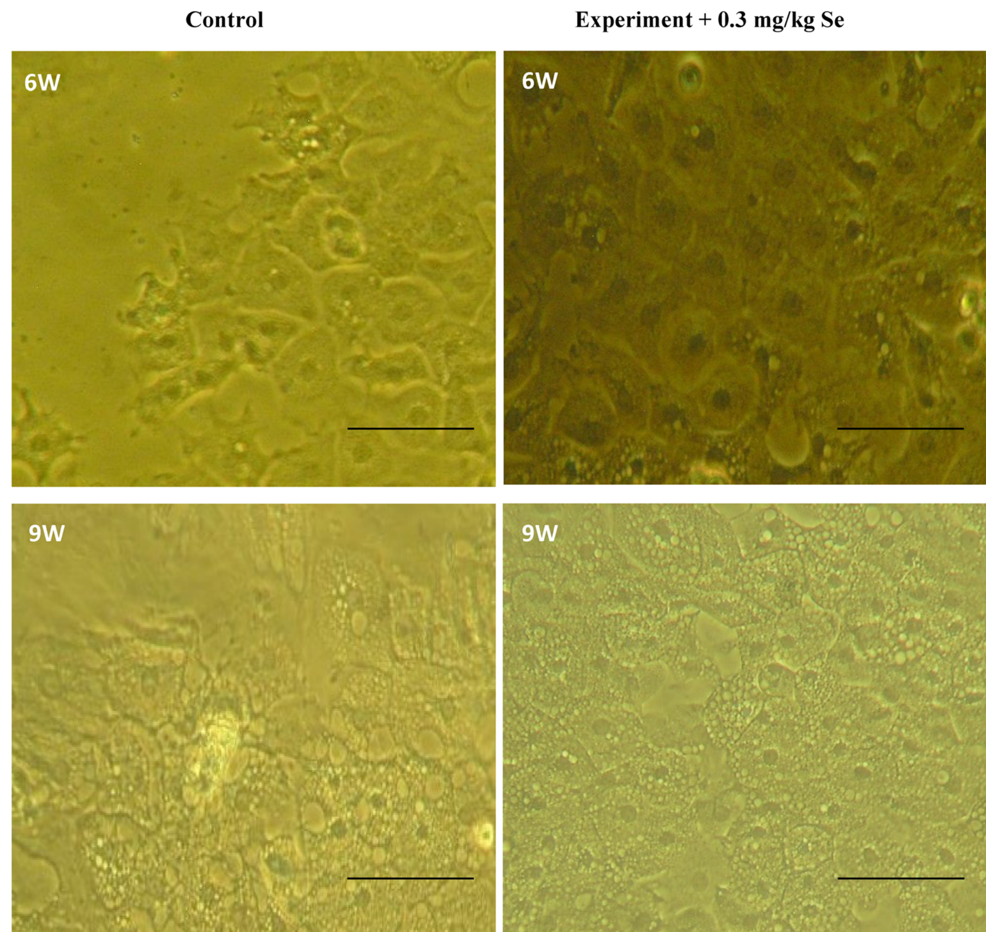

Fig. 3 Sertoli cells from 6- and 9-week-old chickens cultured in vitro in both groups

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Preparation of Testicular Samples for Histological Observations

Testes were cut into 1–2-mm^3 blocks and fixed immediately in 2.5 % glutaraldehyde in 100 mM phosphate buffer at pH 7.0 for 2–24 h. Sections for histological observation were prepared according to the method of Reynolds [20]. Tissue sections from 5 male chickens in each group were used, and a minimum of 60 ($n = 30$) tubules were used to evaluate circular cross-sections. The combined numbers of cells in the circular tubule cross-sections were used as the denominator to calculate cell ratios. The sections were viewed and photographed under a transmission electron microscope (TEM; Hitachi, Tokyo, Japan).

Quantitative Real-Time PCR Analysis

Total RNA was extracted from testes tissues (−80 °C) by using Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. RT-PCR and PCR, to prepare cDNA from this procedure, were used in the following PCR reactions performed with 0.24 μmol/l of each sense and antisense primers, 0.06 mmol/l of rTaq polymerase, 0.8 mmol/l deoxynucleotide mix, and 10× PCR buffer. The PCR reaction was performed as follows: predenaturation for 5 min at 94 °C, denaturation for 30 s at 94 °C, annealing for 30 s at 61.5 °C, extension of 40 s at 72 °C, 30 cycles, and extension of 10 min at 72 °C and 4 °C. The results were analyzed on a 1.5 % agarose electrophoresis gels.

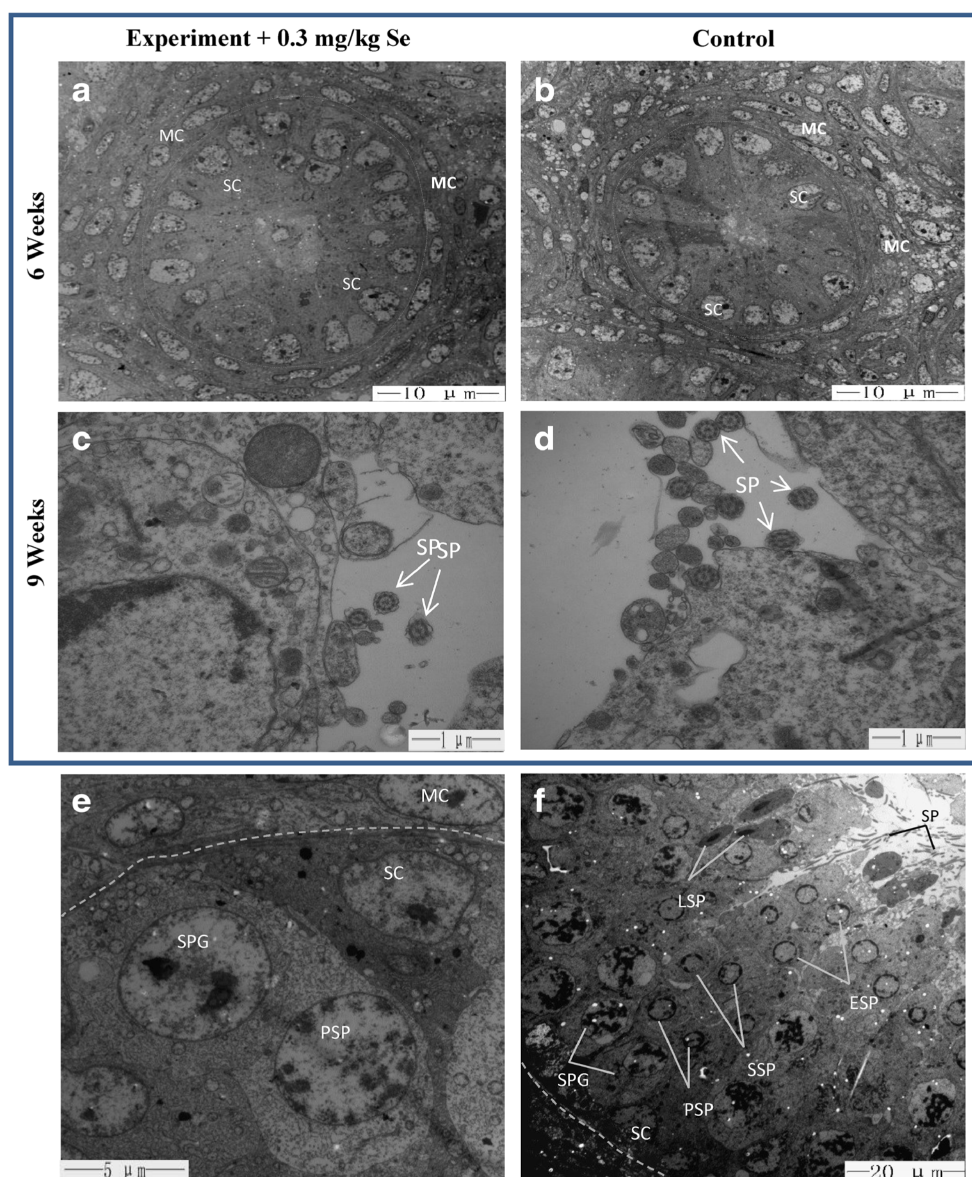

Fig. 4 Transmission electron microscopic (TEM) images of a cross-section of seminiferous tubules from a chicken in the experiment (**a**, **c**) and control groups (**b**, **d**) at 6 and 9 weeks of age, respectively. **e** Seminiferous tubules at 6 weeks show normal testicular architecture and a regular developmental course of spermatogonia, primary spermatocytes, Sertoli cells, myoid cells, and the basal lamina. **f** Seminiferous tubules at 9 weeks show normal testicular architecture and a regular developmental course. Spermatogenic cells = *SP*, late spermatogenic cells = *LSP*, early spermatids = *ESP*, secondary spermatocytes = *SSP*, primary spermatocytes = *PSP*, spermatogonium = *SPG*, Sertoli cells = *SC*, myoid cells = *MC*, basal lamina = *broken line*. *$P < 0.05$ ($n = 30$)

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The quantitative real-time PCR was performed on an ABI PRISM 7500 Detection System (Applied Biosystems, USA). Reactions were performed in a 20-mL reaction mixture containing 10 mL of a 2× SYBR Green I PCR Master Mix (TaKaRa, China), 2 mL of diluted cDNA, 0.4 mL of each primer (10 mM), 0.4 mL of 50× ROX reference dye II and 6.8 mL of PCR-grade water, in condition of 95 °C for 30 s followed by 40 cycles of a 2-temperature program of 5 s at 95 °C and 20 s at 60 °C. β-actin was used as the reference gene and the oligonucleotide sequences of the forward and reverse primers for the target genes are shown in Table 1. The relative quantification was expressed as a ratio of the target gene to control gene using the delta-delta Ct ($^{\Delta\Delta}$Ct) method [21].

Statistical Analysis

The experimental data are presented as mean ± *SD*. The statistical analysis was conducted with CoStat software (CoHort Software, Monterey, CA, USA). Differences between the two groups were detected by one-way analysis of variance (ANOVA) and the *t* test. A *P* value <0.05 was considered significant.

Results and Analysis

Relative Testicular Weight and Seminiferous Tubule Size

Body weight, absolute testis weight, and relative testis weight did not significantly differ during the experiment between the selenium-treated group and the control group. The testis weight was calculated as a percentage of carcass weight without bowels in the control group (27.07 ± 7.7, 58.86 ± 38.65) and in the experiment group (35.49 ± 13.81, 80.4 ± 57.16) at age of 6 and 9 weeks, respectively. The results showed that the right testicle was larger than the left testicle. As well as the total weights between the two groups, there were not significant differences in both ages ($P > 0.05$) (Fig. 1a). Results also showed that there was not any significant difference ($P > 0.05$) in the testicular size tubes between the experiment group (53.66 ± 6.3, 153.70 ± 13.14) and the control group (49.29 ± 8.80, 145.98 ± 21.23) at age of 6 and 9 weeks respectively(Fig. 1b).

Sertoli Cell Viability and Culture

Sertoli cell viability was higher in cells from 6- and 9-week-old chickens in the experimental group than from those in the control group (Fig. 2; $P < 0.05$). The first-passage Sertoli cells from 6- and 9-week-old chickens in the experimental group grew better in vitro than those from the control group (Fig. 3).

Ultrastructural Observations of Testis at Different Ages

Cells in all stages of spermatogenesis, such as spermatogonia, primary spermatocytes, and mature spermatozoa, were found in the seminiferous tubule epithelium (Fig. 4c–e) and were characterized by large ovoid nuclei containing a finely granular nucleoplasm. Nuclei had their long axis parallel to the boundary tissue, the chromatin was homogenous, and the cytoplasm was granular. The primary spermatocytes had spherical nuclei with finely granular nucleoplasm and clumped chromatin. The morphology of the Sertoli cells was normal and was characterized by a cytoplasm that extended from the basal lamina to the lumen of the seminiferous tubules and enveloped the adjacent germinal elements. No degenerative changes or vacuolization was evident (Fig. 4a, b, e). These observations indicate that the testicular architecture and spermatogenesis were normal in both groups at 6 and 9 weeks (Fig. 4e, f).

A greater number of cells per tubular cross-section were detected in the experimental group than that in the control group at 6 weeks (Fig. 5).

Testicular Expression of Se1W, LHCGR, Gpx4, and ACE

Testicular Se1W expression was higher in the experimental group at 9 weeks than that in the control group ($P < 0.05$), and LHCGR and Gpx4 expression levels were higher in the experimental group at 12 weeks than those in the control group ($P < 0.05$). Moreover, ACE expression levels were higher in the control group at 9 and 12 weeks than those in the experimental group ($P < 0.01$; Fig. 6).

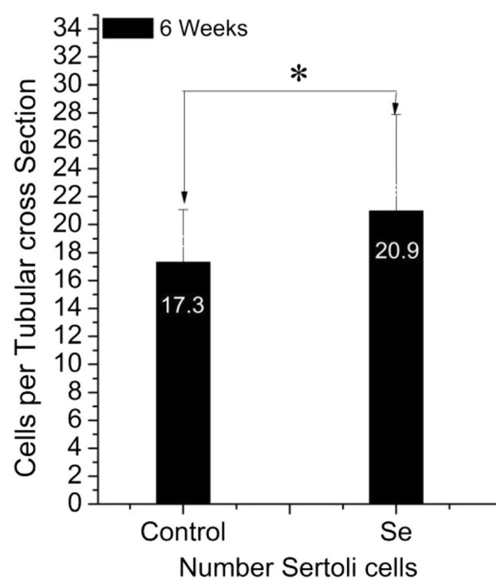

Fig. 5 Mean number of Sertoli cells per tubular cross-section. *$P < 0.05$ ($n = 30$)

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Discussion

Se is essential for normal functioning of the male reproductive system [22]. This is the first study to associate Se levels, Sertoli cell viability, and gene expression. Our results illustrate that dietary supplementation with Se had no effect on relative testicular weight and that testicular ultrastructure, spermatogenesis, and Sertoli cells were normal in both groups. We did observe an increase in the number of cells in seminiferous tubule cross-sections in the experimental group at 6 weeks of age compared to that in the control group, suggesting that Se supplementation enhanced development of the seminiferous tubules at the cellular level. These results correspond to studies that have found enhanced development in the muscular and skeletal systems after Se administration [23]. Se deficiency induces testicular apoptosis [24]. Some studies have reported that Se supplementation does not affect testicular weight [25]. Alternatively, Edens and Sefton [26] reported that broiler breeder roosters supplemented with a slightly lower Se concentration (0.2 mg/kg diet), regardless of source, had increased relative testicular weights than those of the Se-deficient control. However, increased testicular size does not necessarily translate into increased fertility or result in higher reproductive hormone levels and any identified relationships between these measures are breed-specific in poultry. Although testicular size does not seem to be a good phenotypic characteristic to assess the impact of Se on reproduction, a clear relationship has been reported between testicular Se content and sperm quality [27]. Our results show that Sertoli cell viability was higher in the experimental group at 6 and 9 weeks of age than that in the control group and that first-passage Sertoli cells from the experimental group grew better in vitro

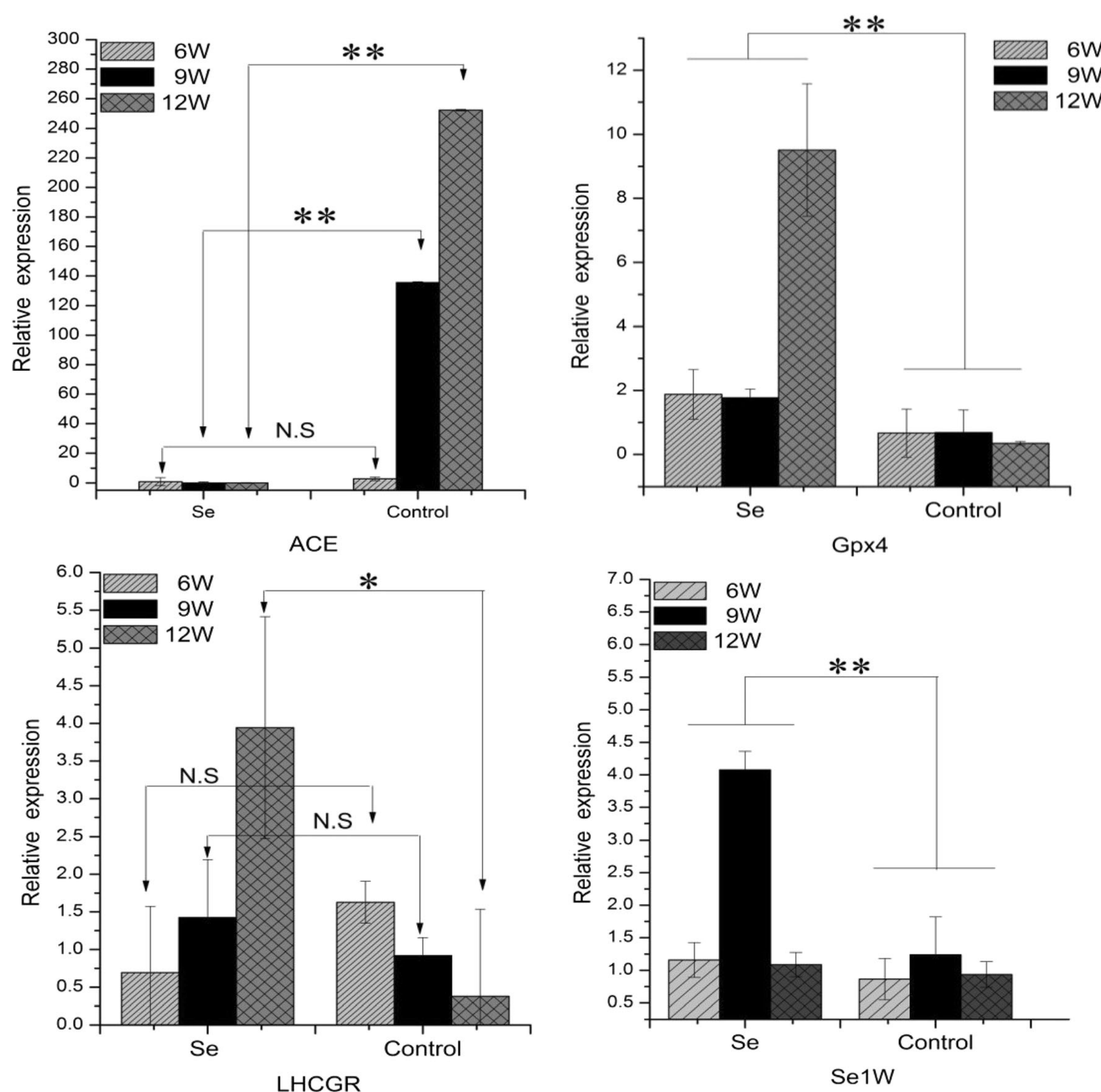

Fig. 6 Relative expression of genes in rooster testes was determined by quantitative real-time polymerase chain reaction analysis. Values are means ± standard error (*SD*) ($n = 3$). $^{**}P < 0.01$, $^{*}P < 0.05$; comparing the selenium experimental group with the control group at 6, 9, and 12 weeks of age

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than those from the control, indicating that Se may improve the microenvironment for spermatogenesis. Se supplementation in young and adult mice improves sperm quality and upregulates testicular genes that are indispensable for proper sperm cell functioning [28].

SelW expression in poultry tissues is sensitive to the concentration of dietary Se [23, 29]. In our study, we found that SelW was regulated by dietary Se content. Many studies have shown that SelW levels increase in response to dietary Se supplementation [30], which is similar to our result. GPx4 is much more resistant to dietary Se deficiency than the other GPx enzymes [31]. Several Sels function as transcriptional regulators by altering the redox status of transcription factors and activating or inactivating gene transcription. We hypothesized that Se supplementation would affect the expression of some testicular genes important for reproduction. The Se-supplemented chickens had higher GPx4 activity in testicular tissues at 12 weeks of age, which supports another report that normal levels of Se present during homeostasis upregulate endogenous antioxidant defenses by increasing GPx activity [32]. It is well known that the GPx4 protein is involved in sperm maturation and has very high activity in the sperm tail structure [33], which confirms a beneficial role for Se in male fertility [34]. Our results show a relationship between Se and GPx4 levels, which corresponded with a previous study [35] that reported a relationship between increased Se and increased GPx4 expression in chicken testicles. It also agrees with another study [36] confirming that chicken tissue GPx 4 levels vary with increased Se levels in food, which was contrary to [33]. We detected relationships between increased dietary Se supplementation and enhanced seminiferous tubule status at the cellular level and increased expression of SelW, GPx4, and LHCGR in chicken testicular tissues.

LHCGR regulates LH and LCG activities. One study showed that disabling the LHCGR gene disables LH and LCG nerve signal transmission, resulting in male infertility, a small-sized penis, and hermaphroditism [37]. Some data indicate that LHCGR affects the adrenal cortex, suggesting a relationship between this receptor and ACE activity. Our findings suggest a hormonal relationship between high LHCGR expression and inhibited ACE gene expression, and this relationship is of interest for increasing male fertility.

Testicular ACE is an ACE isozyme that plays a crucial role in male reproduction. We found decreased ACE expression at 6 weeks but increased expression at 9 and 12 weeks in the control group, indicating that testicular ACE is only present in developing spermatids and mature sperm. In the early 1980s, testicular ACE was found to be absent in immature rats; however, activity of this enzyme increases during puberty, suggesting that its expression is under hormonal control [38]. The finding is in agreement with those of Shibahara et al. [12]. Thus, testicular ACE remains a mystery; particularly its relationship with Se. Our results will allow future studies to investigate the role of Se in ACE expression and its effect on the antioxidant system and levels of superoxide dismutase and ROS.

In conclusion, the basal diet + 0.3 mg/kg Se was beneficial for seminiferous tubule development and promoted Sertoli cell viability. Se was important for regulating expression of the Gpx4, SelW, LHCGR, and ACE genes. These results suggest that dietary supplementation with Se enhances development of seminiferous tubules at the cellular level and involves SelW, GPx4, LHCGR, and ACE.

Acknowledgments This work was supported by the National International Scientific and Technological Cooperation Project (2011DFA30760-2-1) and Fund of Key Lab. NEAU. China (GXZDSYS-2012-07).

Compliance with Ethical Standards All procedures used in the present study were approved by the chicken hatchery, College of Animal Science, Northeast Agriculture University, Harbin, China.

Conflict of Interest None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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