



EFFECTS OF NANO-SELENIUM AND SODIUM SELENITE ON *SELP*, *GPX4* AND *SELW* GENES EXPRESSION IN TESTES OF BROILER BREEDER ROOSTERS

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Summary

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Fertility is the main element affecting hatchability as an important indicator in breeder flocks' performance. Roosters' aging reduces their fertility. Using additives such as selenium helps to delay this reduction through antioxidant properties of Se. Replacing inorganic Se by the nano form in poultry diet improves fertility. The aim of this study was to assess effects of different amount of nano-Se on *Selp*, *Gpx4* and *Selw* gene expression in broiler breeder rooster's testis. A total of 30 fifty-weeks-old broiler breeder males were randomly divided into five groups including a control group (fed basal diet) and four treatments containing basal diet plus 0.3 mg/kg sodium selenite (1), 0.15 mg/kg nano-Se (2), 0.3 mg/kg nano-Se (3) and 0.6 mg/kg nano-Se (4). The results indicated that mRNA expression levels of *Selp*, *Gpx4* and *Selw* genes increased significantly ($P < 0.05$) after dietary supplementation of nano-Se compared to control and sodium selenite groups. In conclusion, dietary supplementation of nano-Se was more effective than sodium selenite on mRNA expression level of tested genes.

Key words: broiler breeder, *Gpx4*, nano-selenium, *Selp*, *Selw*, testis

INTRODUCTION

In commercial broiler breeder flocks, delaying the reduction of male's fertility is an important issue to maintain high reproductive performance. It has been found that phospholipid fraction of avian spermatozoa membranes has high proportion of polyunsaturated fatty acids (PUFA) (Surai, 2002) and that is why the sper-

matozoa are susceptible to free radical attack as well as lipid peroxidation. Therefore, for maintaining sperm fertilising ability, antioxidant protection is a crucial point. The natural antioxidants along with enzymes having antioxidant characteristics (e.g. glutathione peroxidase etc.) form a consolidate antioxidant

system in avian semen protecting sperm against free radicals and their destructive metabolites (Surai, 2002). Semen's fertilising ability can be determined by the elegant equilibrium of antioxidant defense and production of free radicals and correlation between antioxidant protection in avian semen and fatty acid index, also regulating these parameters by nutritional resources (Surai *et al.*, 2001).

To ameliorate male fertility, a great opportunity is to increase antioxidant capacity of semen (Surai *et al.*, 2003). In avian reproduction, antioxidants such as vitamin E and selenium (Se) have significant roles. To achieve great reproductive performances in commercial poultry, antioxidant supplementation at an optimum level seems to be necessary. With this regard Se is an important element of antioxidant system (Surai *et al.*, 2006).

Selenium is a part of at least 25 selenoproteins and chickens have at least 23 selenoproteins (Kaur & Bansal, 2005). SelP is a unique selenoprotein, and the only one known to contain several selenocysteine residues per molecule. More than 50% of plasma selenium exists in SelP, showing the Se transport function of this protein (Yuan *et al.*, 2013). Also, SelP has *in vivo* and *in vitro* antioxidant effects (Schweizer *et al.*, 2005; Peng *et al.*, 2007).

It has been reported that glutathione peroxidase 4 (GPx4) and selenoprotein W protect cells from damage caused by reactive oxygen species (ROS) (Yao *et al.*, 2014). Despite the lack of Cys37 in the peptide, SelW plays an antioxidant role in chickens (Traulsen *et al.*, 2004). In chickens GPx4 mRNA levels are downregulated by excess of Se (Yu *et al.*, 2011).

It has been authenticated that selenium takes part in spermatogenesis. Many studies have been devoted to discover the

main differences between various sources of selenium in the diet. Depending on dietary Se source, there are differences in assimilation, distribution and accumulation of the above element in tissues (Surai *et al.*, 2006). Also, the level of dietary Se regulates the expression of selenoproteins (Tarze *et al.*, 2007).

It has been indicated that replacement of sodium selenite or at least a part of it in the poultry diet by other sources of Se e.g. the nano form, can be helpful to eliminate required selenium and therefore improve fertility and hatchability (Surai *et al.*, 2006).

In poultry industry Se is added to diets as inorganic salts, such as sodium selenite, but there are limiting factors for using them – their bioavailability and toxicity (Karadas *et al.*, 2005). Sodium selenide is a metabolite of sodium selenite that is highly toxic (Ruan *et al.*, 2012). These aspects have persuaded researchers to seek different Se forms with significantly lower toxicity and higher bioavailability and efficacy. Regarding to this, nano-Se has been recently paid more attention and the studies on comparative toxicity and efficacy confirmed its lower toxicity and higher bioavailability seen along with efficient selenoprotein induction (Li *et al.*, 2012; Shi *et al.*, 2014).

Therefore, this study was designed to evaluate differential effects of nano-selenium and sodium selenite on *SelP*, *GPx4* and *SelW* gene expression in broiler breeder roosters.

MATERIALS AND METHODS

Birds, diets and experimental design

Thirty Arbor Acres Broiler Breeder roosters at the age of 40 weeks were randomly assigned to five treatments, each of which were replicated three times with 2 birds

per replicate and were housed in same size pens on a deep litter system. The 1st week of the experiment was adaptation period and the cockles were fed standard basal diet as recommended by Arbor Acres Plus (Anonymous, 2016). Afterwards, feeding of the control group continued with the basal diet (containing 0.3 mg/kg inorganic selenium), but the treatment groups were fed the basal diet supplemented with 0.3 mg/kg of sodium selenite, 0.15 mg/kg, 0.3 mg/kg and 0.6 mg/kg of nano-Se respectively for 4 weeks (Surai & Fisinin, 2014).

Experimental ethics

This study was authorised and the procedures were performed according to the standard animal experimentation protocol of the Veterinary Ethics Committee of Faculty of Veterinary Medicine, Urmia University.

Selenium sources

Two selenium sources that were used in this experiment. The sodium selenite: inorganic form most commonly used form in poultry industry, was purchased from Merck Company, Germany (Art. 6607). The nano-Se (CAS#: 7782-49-2) with 10–45 nm and 99.99% was purchased from American Elements company, USA (<https://www.americanelements.com/selenium-nanoparticles-7782-49-2>).

Sampling and analytical methods

At the end of experiment, the roosters were humanely euthanised by cervical dislocation (Collett, 2013). Afterward testes of all the birds were collected and split into smaller sections, which were snap-frozen in liquid nitrogen, and stored at –80 °C for subsequent molecular analysis.

Selenoprotein gene expression in testicular tissue

For evaluation of the influence of nano-Se on selenoprotein gene expression, the Quantitative Real-Time PCR (qRT-PCR) was used as described below:

RNA Extraction. Total RNA was extracted from frozen testis tissues of breeder males using SinaPure-RNA kit (Cell culture, Tissues, Serum and Plasma. SinaClonBioScience) (Cat.No. PR891620) following the manufacturer's instructions. At the end, the concentration of extracted RNA was measured and recorded using NanoDrop device, an ND-2000 spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, USA) and total RNA was stored at –70 °C until cDNA synthesis.

cDNA synthesis. cDNA was synthesised using Viva 2-steps RT-PCR kit (Vivantis Technologies) (Cat. No.: RTPL12-100app, Malaysia) following the manufacturer's instructions. cDNA Synthesis Mix (M-MLV Reverse Transcriptase + 10× Buffer M-MuLV + Nuclease-free Water) and random Hexamers primers (Primer + dNTP Mix + Nuclease-free water) and 10 µg of total RNA were used by the process of incubation at 42 °C for 60 min and terminating the reaction by incubating at 85 °C for 5 min followed by cooling at 4 °C to synthesise cDNA and it was stored at –20°C until processed.

PCR. PCR reaction was done using Sina clon PCR kit. The PCR procedure consisted of denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and of 60 °C for 20 s (Khalid *et al.*, 2016). The obtained products were electrophoresed in 1.5% agarose gel using a 100 bp DNA marker for verification of primer specificity and product purity.

Table 1. Specifications of the primers used in PCR & RT-PCR

Gene	Primer sequence	Gene size (bp)	Reference
<i>SelP</i>	F: GAGGGACTGGTCAACATCTCATACG R: GGGAAGACCCAGGTGGTACACT	216	Yuan <i>et al.</i> , 2013
<i>GPx4</i>	F: GCCACCTCCATCTACGACTTC R: TCCTTCAGCCACTTCCACAG	335	Khalid <i>et al.</i> , 2016
<i>SelW</i>	F: CTCCGCGTCACCGTGCTC R: CACCGTCACCTCGAACCATCCC	150	Khalid <i>et al.</i> , 2016
<i>GAPDH</i>	F: GCCCAGAACATCATCCCA R: CCAGCACACGCATCAAAG	293	Shi <i>et al.</i> , 2014

SelP: Selenoprotein P, *GPx4*: glutathione peroxidase 4, *SelW*: Selenoprotein W, *GAPDH* = glyceraldehyde 3-phosphate dehydrogenase, F= forward, R= reverse.

Real-Time PCR

To survey the relative amount of *SelP*, *GPx4* and *SelW* gene expression, the produced cDNA samples were used by the quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) technology with StepOne™ Real-Time PCR System (Applied Biosystems) in comparison with *GAPDH* as reference gene. PCR reaction was produced on the basis of final volume of 25 µL that consisted of 12.5 µL ready to use mixture (SinaSYBRBlue HF-qPCR Mix, Cat. No. MM2171, SinaClon), 0.25 µL of each of forward & reverse primers (each 2 µmol; Table 1), 2.5 µL cDNA, 9.5 µL nuclease free water. The procedure comprised a denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and of 60 °C for 20 s (Khalid *et al.*, 2016). All samples were measured in triplicate.

To confirm the effectiveness of PCR reaction with consecutive dilution of cDNA using the tested gene, real-time PCR was performed. The specificity of products was validated using melting curve analysis. A standard curve from cDNA reaction mixture was used to distinguish the efficiencies of PCR, which

were ascertained between 90 and 110 percent. The R2 amounts of all curves were observed from 0.997 to 0.999. To quantify the PCR product, the amounts of CT were used and the relative expression level of the target genes were expressed as 2-CT. Subsequently, the CT was derived from the subtraction of the housekeeping gene (*GAPDH*) CT from the tested gene CT (Shi *et al.*, 2014).

Statistical analysis

The statistical analysis was conducted using SPSS version 23 for Windows (SPSS Inc., Chicago, USA). Average values of relative expression in different groups were compared by one-way analysis of variance (ANOVA). Afterwards difference between groups was investigated by least significant difference (LSD) test. The experimental data are presented as mean ± SD. Differences were considered as significant at P<0.05.

RESULTS

To check the accuracy and ensure that the cDNA product is correct, multiplied products in the polymerase chain reaction with *SelP*, *GPx4* and *SelW* gene primers

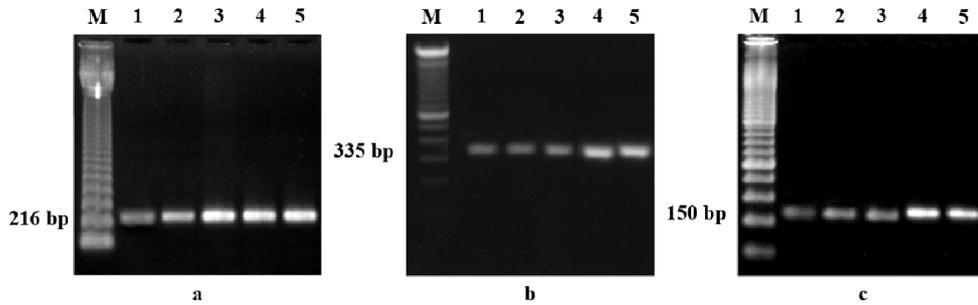


Fig. 1. Bands derived from cDNAs samples PCR using *SelP* (a), *GPx4* (b) and *SelW* (c) genes primers. M: Ladder, lane 1: control group, lane 2: 0.3 mg/kg sodium selenite, lane 3: 0.15 mg/kg nano-Se, lane 4: 0.3 mg/kg nano-Se, lane 5: 0.6 mg/kg nano-Se.

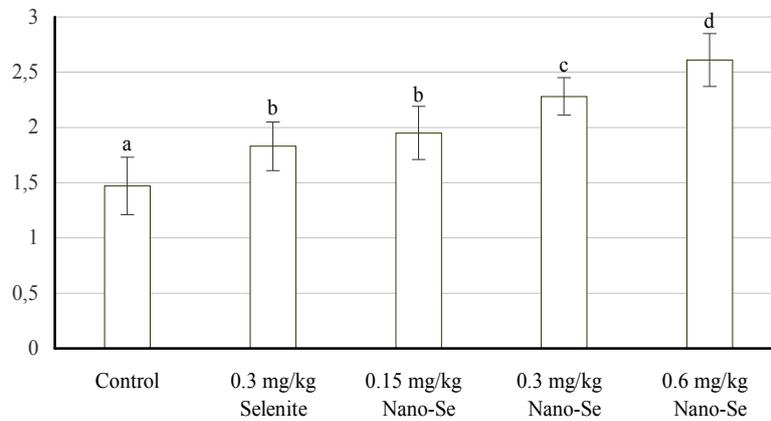


Fig. 2. The mRNA expression level (mean±SD) of the *SelP* gene among different treatment groups relative to the reference gene (fold increase). Significant differences between groups are shown by different letters ($P<0.05$).

were electrophoresed in 1.5% agarose gel using 100 bp DNA (Fig. 1).

mRNA expression of SelP gene

SelP mRNA expression levels in the testis of broiler breeder males were significantly ($P<0.05$) increased as the level of nano-Se supplementation increased. Quantitative evaluation of the *SelP* PCR products revealed that roosters fed diet with 0.6 mg/kg nano-Se had the highest (2.61 ± 0.24) *SelP* mRNA expression level and compared to those of roosters fed control diet (1.47 ± 0.26). All treatments resulted

in significant increase compared to the control group. mRNA expression level in the treatment of 0.6 mg/kg nano-Se was higher than 0.3 mg/kg nano-Se supplementation (2.28 ± 0.17) and the level in 0.3 mg/kg nano-Se fed group was greater vs that with 0.15 mg/kg nano-Se in the diet (1.95 ± 0.24). The treatment of 0.15 mg/kg nano-Se lead to *SelP* mRNA higher expression level than that of birds supplemented with 0.3 mg/kg sodium selenite (1.83 ± 0.22). The increasing trend in *SelP* mRNA expression was seen among groups with significant differences be-

tween them except for *SelP* mRNA expression level of roosters fed diet with 0.15 mg/kg nano-Se, that did not differ significantly from that of roosters fed diet with 0.3 mg/kg sodium selenite (Fig. 2).

mRNA expression of GPx4 gene

Glutathione peroxidase 4 PCR products were used for quantitative evaluation. This gene showed a different pattern of increase in response to the treatments

compared to the other two genes in this study. We found that roosters from 0.3 mg/kg nano-Se and 0.6 mg/kg nano-Se treatments had a significantly ($P < 0.05$) higher *GPx4* mRNA level (0.79 ± 0.15 and 0.86 ± 0.15 , respectively) than those from the control group (0.31 ± 0.07). The 0.6 mg/kg nano-Se treatment had the highest mRNA expression level but no significant difference were found between 0.3 mg/kg and 0.6 mg/kg nano-Se. The

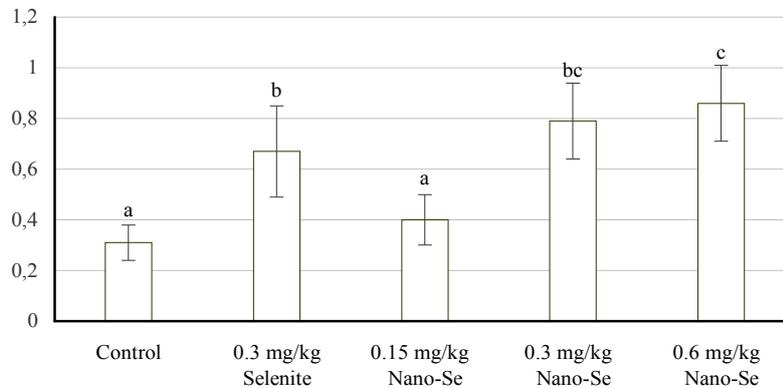


Fig. 3. mRNA expression level (mean±SD) of the *GPx4* gene among different treatment groups relative to the reference gene (fold increase). Significant differences between groups are shown by different letters ($P < 0.05$).

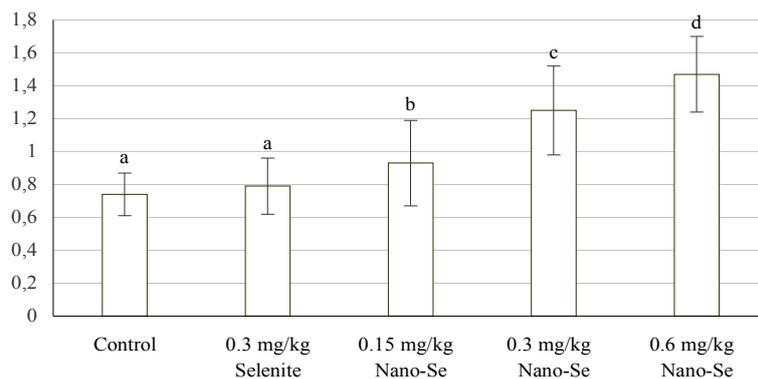


Fig. 4. mRNA expression level (mean±SD) of the *SelW* gene among different treatment groups relative to the reference gene (fold increase). Significant differences between groups are shown by different letters ($P < 0.05$).

mRNA expression level of 0.15 mg/kg nano-Se treatment (0.40 ± 0.10) did not differ significantly compared to the control group while the *GPx4* mRNA level in of sodium selenite treatment (0.67 ± 0.18) showed a significant increase in comparison with controls although not significantly different from expression of the same amount of Se in the nano form (0.3 mg/kg nano-Se) (Fig. 3).

mRNA expression of SelW gene

SelW mRNA expression levels of broiler breeder testis in all three nano-Se treatments (0.15 mg/kg nano-Se, 0. were 0.93 ± 0.26 , 1.25 ± 0.27 and 1.47 ± 0.23 , respectively. They were significantly ($P < 0.05$) higher than that in the control and also from the sodium selenite treatment (0.79 ± 0.17). There was no significant increase in *SelW* mRNA expression level of the group treated with sodium selenite in comparison to control group (Fig. 4).

DISCUSSION

It has been reported that in poultry, the maternal diet influences the hatchability and embryonic mortality (Wilson, 1997; Karadas *et al.*, 2005). According to this, poultry's diverse disorders like impaired fertility, decreased hatchability and also increased embryonic mortality are associated with Se deficiency (Shi *et al.*, 2014). Se is an essential trace element for normal function of male reproductive system (Surai *et al.*, 2006). Gray and black elemental selenium are known to be biologically inert. Thus, feed supplementation with selenium is limited to organic form and also inorganic sodium selenite that is most commonly used in poultry industry. However, bioavailability and toxicity are the factors limiting the use of Se (Li *et al.*, 2012). As a result, a form of Se with sig-

nificant high membrane permeability, lower toxicity and higher efficacy like nano-Se makes the element more feasible to use. Several studies confirm the effectiveness of nano-Se on selenoproteins induction with lower toxicity and improved bioavailability (Peng *et al.*, 2007; Wang *et al.*, 2007). It is thought that the beneficial effects of Se are mediated through the function of selenoproteins (Yu *et al.*, 2011). The importance of Se availability as a regulator of selenoprotein gene expression is reported (Bermano *et al.*, 1995).

The present study investigated the mRNA expression of *SelP*, *GPx4* and *SelW* genes in testis of roosters whose diet was supplemented with various levels of nano-Se compared to a group with the basal diet supplemented with sodium selenite (as inorganic source).

A previous study by Burk *et al.* (1995) reported that *SelP* was expressed in a variety of tissues and it was shown to perform functions in the transportation and delivery of hepatic Se throughout the body (Hill *et al.*, 2003; Schweizer *et al.*, 2005). *SelP* also acts as an antioxidant (Burk *et al.*, 1995). Knowing the way *SelP* is influenced by different forms of dietary Se is important, because the level of this protein may be related to susceptibility to oxidant-induced disease. As shown on Fig. 2, the highest and lowest mRNA expression of the *SelP* was observed after feeding 0.6 mg/kg nano-Se and in the control group, respectively. Also, the expression of *SelP* gene in the group fed 0.3 mg/kg nano-Se was greater than in that treated with 0.15 mg/kg nano-Se, and the expression in 0.15 mg/kg nano-Se fed birds was insignificantly greater than in those fed 0.3 mg/kg sodium selenite group. It has been shown in several researches that *SelP* expression is

highly up-regulated by Se supplementation in comparison with Se deficiency (Gao *et al.*, 2012; Hadley & Sunde, 2001; Hill *et al.*, 1996; Pagmantidis *et al.*, 2005). In agreement with these reports, our study demonstrated a similar enhancement in the expression of this selenoprotein gene.

Normal levels of Se that have been found during homeostasis upregulate endogenous antioxidant defenses due to increasing GPx activity (Foresta *et al.*, 2002). It's been found that *GPx4* protein is involved in sperm maturation and in the sperm tail structure, it has high activity (Zhang *et al.*, 2013), which confirms the great beneficial role of Se for male fertility (Zoidis *et al.*, 2010). We found that the expression of *GPx4* gene was increased parallelly to increasing nano-Se level in the diet and that the greatest expression was obtained with 0.6 mg/kg nano-Se supplementation although there was no significant difference between 0.6 and 0.3 mg/kg nano-Se. The lowest expression of *GPx4* gene was observed in control group.

Unlike the *SelP* gene expression, *GPx4* gene expression in birds treated with 0.3 mg/kg sodium selenite was greater than in those given 0.15 mg/kg nano-Se but similarly to *SelP* gene expression, *GPx4* gene expression of birds receiving 0.3 mg/kg nano-Se was greater than the same amount of selenium in its inorganic form (sodium selenite); however the difference was not significant. Our results suggested that nano-Se had higher bioactivity than sodium selenite in increasing mRNA level of *GPx4* gene in broiler breeder males. These findings may be helpful in understanding the association of the dietary Se forms and expression of *GPx4* gene in antioxidant function. Our findings are consistent with previously reported research that there was a

relationship between increased Se and increased *GPx4* expression in rooster testicles. It is also in line with another study (Bakircioglu *et al.*, 2014), where chicken tissue *GPx4* levels varied with increasing Se levels in diet (Zhang *et al.*, 2013).

Expression of *SelW* in poultry tissues is sensitive to the concentration of dietary Se (Ruan *et al.*, 2012; Schweizer *et al.*, 2005). The RT-PCR results of our research showed that the mRNA expression of *SelW* gene in testes of broiler breeder males was influenced by the dietary Se levels. The present study indicates that mRNA levels of *SelW* in broiler breeder roosters were regulated differently by specific dietary forms of Se, as nano-Se compared with sodium selenite showed greater effects on *SelW* gene expression. It has been shown in many studies that in response to dietary Se supplementation, *SelW* levels increase (Gan *et al.*, 2002). Our results were comparable as they showed that the treatment with 0.6 mg/kg nano-Se resulted in highest mRNA expression of the *SelW* gene and the lowest mRNA expression of the same gene occurred in the control group (C). There was no significant difference between the treatment with 0.3 mg/kg nano-Se and the control group. Besides, the 0.3 mg/kg nano-Se treatment showed a greater *SelW* gene expression than the 0.15 mg/kg nano-Se and the latter in turn resulted in greater expression of *SelW* than the 0.3 mg/kg sodium selenite group. These findings were just like the response of *SelP* gene expression to dietary Se in our different treatment groups.

In conclusion, dietary supplementation of nano-Se was more effective than inorganic sodium selenite on mRNA expression level of *SelP*, *GPx4* and *SelW* genes.

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