Effect of Organic and Inorganic Dietary Selenium Supplementation on Gene Expression in Oviduct Tissues and, Antioxidant Capacity of Laying Hens

Aliyu Muhammad
Universiti Putra Malaysia
Abdul-Alla Dalia
University of Khartoum
Teck Loh
Universiti Putra Malaysia
Henny Akit
Universiti Putra Malaysia
Anjas Asmara Samsudin (anjas@upm.edu.my)
Universiti Putra Malaysia

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Abstract

Some functional genes were investigated for their involvement in egg (eggshell biomineralization) formation and selenoproteins in the oviduct and liver of laying hens fed different organic and inorganic selenium source. A total of 24 hens were selected randomly from the four treatments and slaughter. Uteri and liver tissue samples were collected from hens during the active growth phase of calcification (15 - 20 h post-ovulation) for RT-PCR. The basal diets supplemented with 0.3mg/kg of different organic Se sources and sodium selenite upregulate uterine and selenoproteins mRNA levels. This research reaps the advantage of tissue sampling from specialized segments of the oviduct that consecutively form different egg components. Expression of OC-17 and OC-116, and OC-17 were significantly higher in the uterus and magnum of laying hens, respectively. Their higher expression was observed with organic Se (bacterial selenoprotein or Se-yeast) fed-hens. The results may postulate the efficacy of organic Se in enhancing the expression of functional genes involved in the egg (eggshell biomineralization) formation and selenoproteins compared to inorganic and non-Se supplemented hens. This study proposed the efficacy of bacterial selenoprotein in the upregulation of the uterine genes and hepatic selenoproteins in laying hens.

Introduction

Selenium (Se) is an essential trace element required for numerous physiological functions in animals, ranging from immunoregulatory function [1,2], reproduction [3], and protecting the tissue damage by the maintenance of the antioxidant system [4,5]. The mechanisms in which Se functions solely is applied via Se-containing proteins [6]. Selenocysteine is the major part by which Se exerts its biological role within a living system after incorporation into selenoproteins [7]. Consequently, selenoprotein levels and the yield of selenoprotein mRNA levels depend on Se availability. In chickens, more than 25 unique selenoproteins were recognized and play key roles in their catalytic activity site. Among which were identified in humans and animals include glutathione peroxidases, iodothyronine deiodinases, and thioredoxin reductase [8].

The synthesis of selenoproteins is pretentious by nutritional form and levels of Se supplementation in the diet [9,10]. A substantial number of research reports confirm a positive connection between dietary Se supplementation and the expression of selenoproteins in animal tissues. The GPx activity signals as a biomarker of Se status [11], and the Se-deficient diet downregulate the expression of selenoproteins in broiler muscular stomach [12]. Similarly, Zhang et al. [13] reported downregulated mRNA levels of 14 selenoproteins genes, and 9 selenoproteins genes upregulated in the low-Se diet group, but neither the DIO3 nor SEPX1 mRNA levels was affected in broiler kidney. Excess Se was reported to down-regulate the expression of GPx4 mRNA levels in chicken liver [14]. Moreover, elevation in liver mRNA of SELW1 was observed for broiler fed with Se (Na_2SeO_3)-supplemented diet for 90 days [15].

The Se form and duration of its supplementation significantly influence the reproductive performance in poultry [16,17].It was observed that Se supplementation (< 8 wk) of either form or source has an

insignificant effect on hen reproductive parameters [18,19]. On the other hand, birds supplemented with organic Se for a period beyond 12 weeks enhanced egg production, fertility, hatching performance in broiler breeder hens' [20,21], layers [22,23], and duck breeders [24]. Nevertheless, it is obvious that Se supplementation affects reproductive performance in hens, yet very little is known on how it modulates these effects. Recently, advancements in genomic technologies, mainly using species-specific microarrays could aid investigation and explain how gene expression profiles are affected by nutrients, thus affecting cellular function. It may serve as a tool for providing useful data on how different forms of nutrients modulate their consequences on production and reproductive performance. Laying hen oviduct is consider a conducive environment biologically for egg development and potential fertilization [25]. The biological process involved in mineralization of the hen eggshell is highly complex, and resulted in the efficient calcium mobilization and biomineralization [25], from the bloodstream via the uterine trans-epithelial cells, and finally into the uterine fluid, which bathes the eggshell [26]. Also, the activation of genes in the biological process of calcification is tissue-specific and timely [26,27].

As reported in previous studies, sources of Se and its levels may show diverse responses in metabolic effects in animal tissues [26,28]. Besides, the Se bioavailability of either source and form is determined by the absorption pathways [29]. Different strains of microorganisms can be employed to produced organic Se via microbial reduction pathway. Among which is *Stenotrophomonas maltophilia* (ADS18) isolated from hot-spring water and linked with rich in organic Se-containing proteins which can be applied as Se source in poultry [11,30]. While it is evident that Se can enhance the antioxidant system, scientific data about the effect of this new organic Se source on layers is limited, and to this edge and to our knowledge, there is no published study reported to investigate the effect of bacterial organic Se of ADS18 source on the expression of some uteri genes and selenoproteins in layers. Therefore, the present study was designed to investigate the efficacy of bacterial organic Se of ADS18 source as an alternative organic Se compared to inorganic Se; on mRNA expression of some selected selenoproteins, and uteri genes responsible for eggshell biomineralization in laying hens.

Results

Effects of dietary selenium supplementation on mRNA expression of eggshell matrix and cuticle proteins in uterus.

The relative expression of OC-17 and OC-116 showed significant changes ($p < 0.05$) in mRNA expression profiles between the experimental groups (Fig. 1a). The mRNA expression of OC-17 and OC-116 were higher ($p < 0.05$) in organic Se (T4 or T3) supplemented compared to inorganic Se (T2) and negative control (T1) of laying hens. Expression of OC-17 mRNA was down-regulated in hens received an inorganic Se (T2) diet compared to either hens fed with organic Se (T4 or T3), or negative control (T1). However, OC-116 mRNA expression increased ($p < 0.05$) in organic Se (T4 or T3) supplemented hens than sodium selenite (T2) or non-supplemented (T1) group. Furthermore, the mRNA expression of cuticle proteins (OCX-32 and OCX-36) in the uterus of the laying hens were influenced by dietary Se treatments (Fig. 1b).

The expression of OCX-32 mRNA was up-regulated in only the organic Se treated group (T4 or T3), significantly different in inorganic (T2) and non-Se supplemented (T1) group. The mRNA expression of OCX-36 was highest in Se-yeast (T3) group followed by bacterial selenoprotein (T4), and sodium selenite (T2) hens, while least in non-Se supplemented (T1) laying hens, respectively.

Effects of dietary selenium supplementation on mRNA expression of eggshell matrix and cuticle proteins in magnum.

The effect of dietary Se supplementation of eggshell proteins gene expression in the magnum of laying hens measured by real-time PCR is shown in Fig. 1c, d. The expression of OC-17 mRNA was up-regulated ($p < 0.05$) only in hens supplemented with bacterial organic Se (T4) (Fig. 1c). However, there was a down-regulation of the same gene in hens fed Se-yeast (T3) and sodium selenite (T2) with no significant ($p > 0.05$) between them, and inferior to the non-supplemented (T1) group. The OC-116 mRNA was down-regulated with all the dietary Se treated groups compared with the non-Se supplemented group. Despite the down-regulation of OC-116 mRNA with bacterial selenoprotein group (T4), yet not statistically different ($p > 0.05$) from negative control (T1), however, both (T1 and T4) are superior ($p < 0.05$) than (T3) and (T2), respectively. Moreover, the results of the quantitative expression of OCX-32 and OCX-36 in magnum showed that the mRNA levels were influenced by dietary Se treatments (Fig. 1d). The expression of OCX-32 mRNA was up-regulated in all dietary Se treatments with the highest expression in bacterial organic Se (T4). However, there was no significant ($p > 0.05$) increase in OCX-32 mRNA expression between Se-yeast (T3) and sodium selenite (T2) fed hens, but superior ($p < 0.05$) than negative control hens (T1). No significant ($p > 0.05$) difference was observed among all the dietary Se treated groups (T2 – T4), but significantly higher ($p < 0.05$) from non-Se supplemented (T1) hens of OCX-36 mRNA expression.

Effects of dietary selenium supplementation on mRNA expression of hepatic selenoproteins in the liver of laying hens.

The relative expression of some selenoproteins of hens supplemented with organic and inorganic dietary Se sources is shown in Fig. 2a, b. The hepatic expressions of GSH-Px1, GSH-Px4, DIO1, DIO2, TXNRD1, and SELW1 genes were studied. The expression of GSH-Px1 and GSH-Px4 (Fig. 2a) was influenced by dietary Se supplementation. Organic Se (T4 or T3) supplemented hens showed higher expression of both GSH-Px1 and GSH-Px4, with a significant difference ($p < 0.05$) than the inorganic and non-supplemented groups, respectively. A significant difference ($p < 0.05$) was higher in T4 than T3, and other treatment groups for GSH-Px1. Similarly, organic (ADS18 or Se-Yeast) Se showed higher ($p < 0.05$) fold changes in mRNA expression of GSH-Px4 than inorganic (sodium selenite) and control group, respectively. However, hens who received a diet supplemented with inorganic Se (T2) also showed an increase in GSH-Px1 and GSH-Px4 mRNA expression compared to the negative control, although not statistically different ($p > 0.05$).

The fold changes in the relative expression of DIO1 and DIO2 mRNA levels in the liver tissue are shown in Fig. 2b. Higher fold change in relative gene expression of both genes was observed in T4 (ADS18) with

significant differences among other treatment groups. Only bacterial selenoprotein (T4) was shown to be significantly ($p < 0.05$) different compared to other treatment groups, however, T3 and T2 were slightly above the negative control but not significantly different ($p > 0.05$) in mRNA expression of DIO1. Similarly, a significant increase ($p < 0.05$) in the DIO2 mRNA expression was observed in the liver of hens supplemented with bacterial organic Se compared to hens fed Se-yeast (T3), sodium selenite (T2), and negative control (T1), respectively. However, the Se supplemented groups hens recorded higher fold changes in the liver mRNA expression of DIO1 and DIO2 regardless of Se forms.

The mRNA expression of TXNDR1 and SELW1 genes trialed livers of hens fed different forms of dietary Se was shown in Fig. 2c. The significant difference in TXNDR1 mRNA fold changes was discerned in the liver of the T3 (Se-yeast) treatment group and significantly ($p < 0.05$) differed with negative control only. However, there was no significant ($p > 0.05$) difference noted in the liver mRNA levels among all the dietary Se supplemented groups. Furthermore, a significant fold change of hepatic SELW1 mRNA level was found in all the Se supplemented groups, with no influence compared to the basal diet with no Se supplementation. However, hens received bacterial organic Se (T4) proved a significant difference ($p < 0.05$) compared to other treatment groups, nonetheless superior fold change in Se supplemented group (T2, T3, and T4) than the negative control group (T1).

Discussion

One of the most critical roles of the eggshell is to enfold and shelter the egg contents through its mechanical properties for optimum economic success in layer production [31]. The eggshell ultrastructure of hen is a highly ordered structure with unique mechanical properties crystal morphology and organic matrix [32,33]. Calcium carbonate contains 95% in its calcitic polymorph and 3.5% macromolecules of the organic matrix. Among many factors that determine its formation are physiological changes and the complex stages of egg calcification involved uterine cells and fluid constituents [32,26]. The formation of complex bio-ceramic eggshell arises from direct acellular uterine fluid interaction of ions such as Ca^{2+} and HCO_3^- and precursors of organic constituents [32,34], with an uninterrupted action of cells [35]. Soluble precursors like proteins and minerals were released by uterus cells into the acellular uterine fluid [33]. A solid layer is formed as a result of interaction between the developing crystal and organic shell matrix with greatly systematic microstructure and texture as eggshell by extraordinary mechanical properties [32,26]. The current study compared gene expression in the oviduct of laying hens supplemented with dissimilar Se sources. Egg formation and yolk ovulation are stimulated by reproductive hormones either during the active calcification stage, thus, regulating the calcium metabolism [33]. Furthermore, genes either connected to the biomineralization process and, or supply the shell precursors could be upregulated. The chicken uterus plays a key role in the daily calcification of the shell during 19 hours process though the egg remains in there. Moreover, a compact layer is formed due to the interaction between the developing crystal and organic shell matrix with largely systematic microstructure and texture as eggshell by great mechanical properties [32,34]. The proteins potentially involved in the biomineralization process were mainly focused on in this study. Numerous research has demonstrated

their roles by interactions between these proteins and crystal formation [26]. However, studies on different Se sources on the expression of reproductive genes were not wholly researched, and to the best of our knowledge, there was no published data reported on the efficacy of bacterial organic Se on laying hens. In the current study, dietary Se supplementation affects the mRNA expression of all the examined genes by either up or down-regulation depending on the type of tissue. Physical egg quality factors such as shell thickness, egg shape, and elasticity are determined by the mRNA expression of OC-116 jointly with OCX-32 genes [32,36]. Devoid of these matrix proteins could result in the cessation of the mineralization process completely [37]. Fragile, shape stiffness, and thickness of eggshells are connected to irregular OC-116 gene expression or OC-116SNP variants [38]. Yin et al. [39] observed the gene expression in the oviduct (magnum and uterus) of pre-laying and laying hens were primarily involved in the growth and development, and the progress of egg formation, respectively.

The chicken ovocleidin gene (OC-17 and OC-116) is expressed in the uterus as one of the potential eggshell matrix proteins [25,37]. They were both characterized as soluble and insoluble eggshell matrix proteins. Both were described during the mineralization process as framework proteins to align calcite crystals [32]. The primary function of OC-17 protein is an antimicrobial and regulates the biomineralization process [40,41]. Ovocleidin-116 is a major component of the chicken eggshell matrix extracellular phosphoglycoprotein, plentiful in uterine fluid during the active phase of calcification stage, and consequently is suspected to function in the process of eggshell mineralization [42,43,44]. Hincke et al. [42,46]established OC-16 function in eggshell and bone strength mineralization, and Sah et al. [25] reported that it regulates the arrangement of calcite crystals in eggshell. It is noteworthy, that the findings showed higher mRNA expression of OC-17 and OC-116 in the uterus tissue than in magnum, regardless of the treatment groups. This was confirmed by published data from [31],[47],[37],[33] who reported higher mRNA expression of OC-116 genes in the uterus tissue. On the contrary, Yin et al. [31] observed OC-17 is differentially upregulated in the isthmus than ovary and magnum, however lower than the uterus. Furthermore, in chicken uterine fluid, there has been an interaction between organic matrix and inorganic minerals resulting in tough and calcification of eggshell [37].

The shell of an egg is considered a physically protective wall for the contents against external (microbial) inversion rich in proteins with antimicrobial properties [33]. These antimicrobial properties are contained in the liquid egg-white, and potentially present chemical defensive mechanisms to the egg [48]. The lumen is sheltered from bacteria-free, thus protect the forming egg or embryo by antimicrobial proteins released into the uterine fluid. Among the examined antimicrobial proteins expressed in the tissues during the shell, calcification includes ovocalyxin-32 and 36. Ovocalyxin family (OCX-32 and OCX-36) constitutes mostly organic matrix proteins [26,33], highly expressed by uterine glandular cells, eggshell membrane and, egg vitelline membrane especially during the active calcification phase [49]. Ovocalyxin-32 is the main determinant of eggshell quality in avian species whereas ovoaclyxin-36 presents an antimicrobial property integrated into the eggshell [50]. Also, antimicrobial properties were discovered in the recombinant of OCX-32 [51]. Besides, OCX-36 belongs to the lipopolysaccharide-binding proteins and Bactericidal

Permeability Increasing (BPI) family and recognized in mammals for its participation in anti-bacterial defense [33]. Members of these family might be lethal to Gram-negative bacteria via binding to the lipid A portion of the lipopolysaccharide cell wall. The present study note that mRNA expression of OCX-32 and OCX-36 were up-regulated in the uterus than magnum tissue irrespective of the dietary Se treatment. In conformity with our results, [47,31] reported highly expressed OCX-32 precursors in the uterus with egg. Similarly, [52] observed a higher expression of the OCX-32 gene in the distal oviduct (isthmus and uterus) and proximal oviduct (magnum and uterus), thus, confirm its secretion from the glandular epithelium of the shell gland [53]. Similarly, Jonchère et al. [53] and Brionne et al. [33] established that OCX-36 is shell gland specific, and increase through the calcification of eggshell. On the other hand, OCX-32 was highly expressed in isthmus than ovary and magnum, although lower than the uterus [31]. Similarly, there is a discovery of OCX-36 expression in isthmus and uterus and expected to participate in natural defense mechanisms because of its similarity to lipopolysaccharide-binding proteins and bactericidal permeability-increasing protein [31]. The findings of Hrabia et al. [54] suggest that growth hormones may participate in the development and activity, and expression of some oviduct specific proteins (OCX-32 and OCX-36) in the chicken. Further studies, however, are required to elucidate the fundamental mechanisms behind these responses.

The present study investigated the expression of selenoproteins in the liver of laying hens fed with two forms of organic Se from bacteria and yeast compared with an inorganic source (sodium selenite). The main organ and site primarily for nutrients (carbohydrate, protein, and fat metabolism) homeostasis is the liver [55]. The up-regulation and down-regulation of selenoproteins mRNA expression is dietary Se ingestion dependent [56]. Selenium has been reported to exert its physiological and biological roles primarily mediated via the activity of selenoproteins [15,57], and lead to chemical and biological dysfunction with its deficiency [57]. In the current study, the mRNA levels of the hepatic selenoproteins in hen's liver significantly upregulated in organic Se compared to inorganic or non-supplemented group, which implied that organic Se may exhibit antioxidant properties, and ultimately reduced oxidative stress [58]. Contrary to inorganic Se, it is passively absorbed into the system with typical lower absorption rates [59]. Those results are consistent with previous studies. The foremost selenoproteins discovered and abundant in the liver are GPx (GPx1-4), which reserves enzymatic properties with the majority being involved in peroxides catabolism [60]. Hou et al. [61] reported Se-enriched *Saccharomyces cerevisiae* (SSC) supplementation significantly increased GPx-1 and GPx-4 expression levels in broiler chicken muscle compared with control, *Saccharomyces cerevisiae*, and sodium selenite group. Recently, Chen et al. [62] reported selenide chitosan sulfate (Se-CTS-S) up-regulate GPx-1 and GPx-4 mRNA levels in hepatocytes and liver of chickens compared with chitosan (CTS), chitosan sulfate (CTS-S), selenide chitosan (CTS-Se), and sodium selenite (Na_2SeO_3). Meng et al. [4] observed higher GPx1 and GPx4 mRNA levels in the liver of laying hens supplemented with nano-Se and Se-yeast, respectively. Wang et al. [63] reported dietary Se-yeast supplementation caused an up-regulation of selenoproteins gene expressions in the liver (10) and muscles (11) of rainbow trout (*Oncorhynchus mykiss*). Similarly, organic bacterial Se showed a significant increase in liver mRNA expression of GSH-Px1, GSH-Px4, DIO1, and TXNDR1 compared to

sodium selenite supplemented broilers (Dalia et al., 2017). Chen et al. [65] reported higher expression of GPx-1 and GPx-4 mRNA levels with organic Se supplementation of Se-enriched *Saccharomyces cerevisiae* compared to other groups in Arbor Acres broilers. Khan et al. [66] observed the upregulation of mRNA expression of GPx1 and GPx4 and downregulation of heat shock proteins genes (HSP60, HSP70, and HSP90) in the chicken heart with Se-enriched probiotics. Luan et al. (2016) observed lower selenoproteins transcript levels in chicken erythrocytes fed a Se-deficient diet, though, with high expression of GSH-Px, TXNDR1, selenoprotein P1 (SELP), and selenoprotein synthetase (SPS2) compared to other selenoproteins. GSH-Px family (GSH-Px1, GSH-Px2, GSH-Px3, and GSH-Px4) are plentiful in the liver catabolizing peroxides as their functions. For instance, GPx1 is a potential antioxidant enzyme with a significant role in the detoxification of lipid hydroperoxides and H_2O_2, whereas GPx4 inhibits atherosclerosis by reducing oxidative stress [66]. It has been reported by previous data that GSH-Px and SELW1 mRNA levels increased with dietary Se intake in poultry [57], and sheep [68]. However, studies on dissimilar Se sources on the expression of these genes was not completely explored, as well as no findings on the efficacy of bacterial organic Se on laying hens. The bioavailability of Se sources or forms and levels differs with the type of tissue and animal species with regards to absorption, deposition, and metabolism could directly or indirectly alter the antioxidant enzyme activities [69]. Therefore, it might be attributed that ADS18 or Se-yeast supplementation is connected to the regulation of GSH-Px's, thus reduced body oxidative stress through the transcription level of GPx1 and GPx4 mRNA in laying hens' liver. It could perhaps by the results obtained shows superior sensitivity of GSH-Px1 than GSH-Px4 to regulation by Se status, and different responses to the mRNA expression to dietary Se between both selenoproteins might differ. Moreover, it can protect from Se-deficiency disorder [70]. Furthermore, mRNA expression of GPx1 and GPx4 can be employed as molecular biomarkers for evaluating Se status as well as the requirements [71].

The iodothyronine deiodinase (DIO) family plays an essential role in thyroid metabolism [72], and thioredoxin reductase (TrxR) genes which constitute a major cellular redox system in all living organism [73]. Furthermore, the qPCR analysis revealed that relative higher mRNA levels of DIO1, DIO2, TXNDR1, and SELW1 genes were expressed in the liver as well, an organ more responsive to changes in the levels and form of dietary Se [74]. As proved by previous studies, [75,74], the findings confirmed that Se sources and intake alter the mRNA levels of laying hens selenoproteins, and the effects vary greatly between different selenoproteins and tissues, although only liver tissue was investigated in this study. Lin et al. [75] reported downregulation of DIO1, DIO2, DIO3, TXNRD2 selenoproteins induced by Se deficiency in chicken's thyroid gland. Liu et al. [76] observed downregulation of the SEPW1 mRNA level in pig's liver fed a high-Se diet of 3.0 mg Se/kg against the 0.3 mg Se/kg diet. Similarly, hepatic expression of GPX1, SEPW1, and SEPW15 mRNA levels were decreased by dietary Se deficiency in chicks liver and muscle (Huang et al., 2011), SelW in layers liver only [15]. Conversely, supplementation of the inorganic form of Se (sodium selenite) leads to higher mRNA expression of GSH-Px1, SELW1, SEP15, and TXNRD1 levels in lamb liver, where GSH-Px 4 was unaffected by the treatment [74]. The results showed that DIO1, DIO2, TXNRD1, and SELW1 transcripts were upregulated in all the liver of Se supplemented groups of hens. In agreement with these

findings, TXNRD and GPX were observed to function in reducing free radical-mediated peroxidation and redevelopment post-Se supplementation to male Wistar rats [77]. Accordingly, higher Se supplementation may be responsible for preserving optimal activities of GPX and TXNRD, and partial detoxification against the negative effects of Cd in male rats [78,79], and broilers [80]. A recent trial on the toxicity of Pb revealed that Se might alleviate the downregulation of GPX4, 2 and 1, DIO1, DIO2, TXNRD2-3, selenoprotein U, I, O, M, K, W, T, S 15 Sepx1, and Sepn1 expression in chicken cartilage tissue [81,82]. Similar results with Se-yeast and SeMet as organic Se sources upregulate GSH-Px1 and TXNDR1 mRNA expression in broiler breeders compared with sodium selenite [28,83]. Furthermore, SELW1 may participate in the protective role against H_2O_2, oxidative stress, and metabolic pathways [84,74,82]. Comparable data were published in rat testes [85], and pig liver [57]. It is noteworthy, that the findings showed a definite trend of up-regulating selenoproteins (GSH-Px1, GSH-Px4, DIO1, DIO2, and SELW1) mRNA expression significantly ($p < 0.05$) with bacterial organic Se supplementation, except for TXNDR1 with Se-yeast hens compared to the negative control. Furthermore, the findings suggested that DIO2 mRNA may be more sensitive to regulation by bacterial organic Se status than others, and perhaps the different response of mRNA expression to dietary Se source might occur between selenoproteins (GSH-Px1, GSH-Px4, DIO1, DIO2, TXNDR1, and SELW1). The noted variance between organic and inorganic Se could be attributed to the higher bioavailability of organic forms, thus, stimulate more selenoproteins gene expression [86]. Similarly, Surai et al. [87] and Meng et al. [4] suggested the mechanisms of action behind nano-Se are by the mediation of the gut microbiota in converting nano-Se into selenite, H_2Se, or Se-phosphate with the synthesis of selenoproteins. It has been established that organic Se compounds such as; SeMet, SeCys, and Se-methyl-Se cysteine among others differ in terms of their bioavailability to the body [88]. Moreover, the current study notes a significant change in mRNA expression of liver selenoproteins in the hens regardless of Se source or form. Though, the mechanisms behind how dissimilar Se sources can regulate the expression of selenoproteins are yet uncertain and required further exploration.

In conclusion, the current study showed that the expression of uterine genes and selenoproteins was upregulated by basal diets supplemented with 0.3 mg/kg of different organic sources of Se and sodium selenite. Compared to inorganic and non-Se supplemented hens, the bacterial selenoprotein proves stronger at increasing the expression of functional genes involved in the formation of eggs (eggshell biomineralization) and selenoproteins.

Materials And Methods

Animal Ethics and husbandry management.

All procedures concerning animals' care, handling, and sampling were carried out in compliance with the ARRIVE guidelines. The study was also performed strictly per guidelines and the regulations under the approval of the Institutional Animal Care and Use Committee of the Universiti Putra Malaysia (UPM/IACUC/AUP-R063/2018). Lohman Brown hens were purchased from commercial layer farm in Selangor, Malaysia at 18 weeks of age acclimatized for four weeks in the research cages. Hens were

caged individually in pens (30 cm × 50 cm × 40 cm, width depth height), assigned randomly into four equal groups, each contained six replicates (6 hens per replicates) under 16-h light and 8-h dark lighting regimen, light beginning at 17:00 local time per Lohman management guide (2018). Before the commencement of the experiment, average egg production (86.81%) and initial live weight (1714±185 g) did not differ among treatment groups. Hens were fed twice daily (08:00 and 15:00), and allowed *ad libitum* access to water and treatment diets during the experimental period. Eggs were collected from egg trays between 17:30 to 18:30 and the individual egg weight of each hen from each replicate was weighed and recorded daily [89].

Experimental Diets.

A corn-soybean meal basal diet (Table 1) was supplemented with 0 (Control diet), basal diet plus 0.3 mg/kg feed inorganic sodium selenite (Na_2SeO_3) (SS), basal diet plus 0.3 mg/kg selenium yeast (SY) and basal diet + 0.3 mg/kg bacterial enriched protein. The four treatment diets were formulated with adequate nutrients required for laying hens according to the National Research Council (1994) and feeding standard of Lohman management guide, 2018. The feeding length was 17 weeks, starting from the time when the birds were 23 weeks of age and ending when they were 40 weeks of age, with the addition of four weeks for acclimatization before the commencement of the studies.

Samples and data collection

Collection of tissues.

A total of 24 hens were selected randomly from the four treatments (a bird from each replicate), slaughter, and dissected. Before slaughter, hen's abdominal palpation was used to assume the egg presence in the uterus. The carcass was skinned ventrally, and uteri samples were collected from hens at the active growth phase of calcification (15 - 20 h post-ovulation) for RT-PCR. It is aimed at targeting higher expression of genes responsible for eggshell biomineralization an egg. Sections of the uterine tissues were scrapped for total RNA isolation, transferred into 5 ml capped tubes, and immediately snapped frozen in liquid nitrogen and stored at -80^{0}C before extraction of RNA. Furthermore, a portion of liver was cut, wrapped in aluminum foil paper for antioxidant enzymes genes and activity assay snapped freeze in liquid nitrogen before storage in -80^0 for RNA extraction.

Total RNA isolation and purification.

Total RNA was isolated from frozen tissues (uterus, magnum and liver) (30 mg) using Rneasy® Mini Kit (Cat. No. 74104, Qiagen, Hilden, Germany) according to manufacturer's instructions. The purity and concentration of total RNA was determined by using Thermo Multiskan® GO (Thermo, USA) and only samples with an RNA quality > 1.9 were further used for quantitative real-time PCR.

Quantitative real-time RT-PCR (qPCR).

The real-time polymerase chain reaction was performed with the Bio-Rad CFX Manager™ 3.1 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA), in 96-well optical reaction plates. Primers used were designed (*HuaGene*™, MyTacg Bioscience Malaysia) according to published *Gallus gallus* sequences Table 2. The synthesis of first strand cDNA was run by reverse transcription of 1 µg isolated total RNA (20 µl reaction mixture) using QuantiNova Rev Transcription Kit (cat. No. 205413, Qiagen, Hilden, Germany). The reaction was done in a Bio-Rad thermal cycler (MyCycler, Germany). Master mix was prepared as per the manufacturer's protocols. Real-time PCR was then performed using QuantiNova SYBR Green PCR Kit (cat. No. 208054, Qiagen, Hilden, Germany) on a Bio-Rad CFX Manager™ 3.1 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Each reaction (20 µL) contained 10 µL QuantiNova SYBR Green Master Mix, 1 µL of each forward and reverse primers, 7 µL of nuclease-free water and 1 µL of cDNA. The qPCR reactions were carried out following standard cycling mode as per kit protocol. Three tissue were used from each hen to determine their stable house-keeping genes using glyceraldehyde 3-phosphate dehydrogenase (GAPDH), beta-actin (β-actin) and TATA-Box Binding Protein (TBP). The target genes were analyzed in duplicates and their expression level was determined using cycle threshold (Ct) values following standard curve method after normalization with reference genes. Genes of interest were amplified through the following thermo cycling program: reverse transcription at 95 °C for 10 minutes, first denaturation at 95 °C for 2 minutes, then 40 cycles of denaturation at 95 ^{0}C for 5 s, and combined primer annealing/extension at 60 ^{0}C for 10 s. The fluorescent data were acquired at the end of each annealing step during PCR cycles with a construct of melting curve to assess the specificity of PCR amplification. A real-time PCR was run for each pair of primer in which cDNA samples were replaced with distilled water to ascertain the absence of exogenous DNA. The efficiency of amplification was determined for each primer pair using cDNA serial dilutions utilization. The fold changes for each target gene was calculated using power of 2 $^{(-\Delta\Delta CT)}$ method described by [90]. The fold changes in data were shown as mean ± standard error. The values were subjected to one-way analysis of variance (ANOVA) followed by Duncan multiple range test for mean comparisons to determine significance at P-value < 0.05 on a SAS (Statistical Analysis System, Version 9.4). The result was compared and presented as a fold change between treatments and the control group.

Statistical analysis.

For reference gene validation, relative expression levels of all the target genes were calculated by the comparative $2^{-\Delta\Delta Cq}$ approach [91,90], in Microsoft Excel (2016), using the two most stable reference genes (GAPDH and β-actin). From the Excel, normalized relative quantities (NRQ) values were further analysed with One-way analysis of variance (ANOVA) using the Proc GLM procedure of SAS software (SAS Institute Inc., Cary, NC), and Duncan Multiple Range Test was used to separate level of significance ($P < 0.05$) between the treatment means. The results were presented as mean ± SEM.

Declarations

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Authors contributions

A. I. M., A.M.D., T. C. L., H. A., and A. A. S., contributed to the original idea and design of the study. A.I. M. conducted the experiments and collected the data. All authors were involved in the manuscript preparation and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

Additional Information

Competing interests: The authors declare that they have no competing interests.

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Tables

Table 1: Ingredient Composition and Calculated Nutrient Levels of the Basal Diet (on Dry Matter Basis)

Ingredients	Con	Na_2SeO_3	Se-Yeast	ADS18
Corn (QL)	44.00	44.00	44.00	44.00
Soybean Meal (QL)	29.00	29.00	29.00	29.00
Wheat Pollard (QL)	11.00	11.00	11.00	11.00
CPO (QL)	3.50	3.50	3.50	3.50
L-Lysine	0.10	0.10	0.10	0.10
DL-Methionine	0.25	0.25	0.25	0.25
Dicalcium Phosphate (18%)	2.00	2.00	2.00	2.00
Calcium Carbonate	7.70	7.70	7.70	7.70
Choline Chloride	0.10	0.10	0.10	0.10
Salt	0.35	0.35	0.35	0.35
Mineral Mix*	0.60	0.597	0.597	0.597
Vitamin Mix**	0.60	0.60	0.60	0.60
Antioxidant***	0.40	0.40	0.40	0.40
Toxin Binder****	0.40	0.40	0.40	0.40
Sodium Selenite	0.00	0.003	0.00	0.00
Se-Yeast	0.00	0.00	0.003	0.00
ADS18-Bacteria	0.00	0.00	0.00	0.003
Total	**100**	**100**	**100**	**100**
Calculated composition				
Metabolizable energy Kcal/Kg	2761.24	2761.24	2761.24	2761.24
Protein (%)	17.66	17.66	17.66	17.66
Fat (%)	5.3	5.3	5.3	5.3
Fiber (%)	3.98	3.98	3.98	3.98
Calcium (%)	3.65	3.65	3.65	3.65
Total Phosphorus (%)	0.88	0.88	0.88	0.88
Av. Phosphorus for poultry (%)	0.48	0.48	0.48	0.48
Analysed Se (mg/kg)*****	0.03±0.01	0.31±0.02	0.32±0.01	0.33±0.02

*Mineral premix provided (per kg of diet): Iron 120 mg, Manganese 150 mg, Copper 15 mg, Zinc 120 mg, Iodine 1.5 mg, and Cobalt 0.4 mg.**Vitamin premix supplied (per kg of diet): Vitamin A (retinyl acetate) 10.32 mg, Cholecalciferol 0.250 mg, Vitamin E (DL-tocopherol acetate) 90 mg, Vitamin K 6 mg, Cobalamin 0.07 mg, Thiamine 7 mg, Riboflavin 22 mg, Folic acid 3 mg, Biotin 0.04 mg, Pantothenic acid 35 mg, Niacin 120 mg and Pyridoxine 12 mg. ***Antioxidant contains butylated hydroxyanisole (BHA). ****Toxin binder contains natural hydrated sodium calcium aluminum silicates to reduce the exposure of feed to mycotoxins. Feed live International Software (Nonthaburi, Thailand) was used to formulate the diets. ***** The Se content measured using ICP.MS.

Table 2: Sequence of genes and primers used for relative quantification by real-time PCR (qPCR) in hen's uterine and liver tissues

Name of Target gene	Nucleotide sequence of primers (5′ → 3′)	Fragment Size (bp)	Reference (s)
Oviduct genes			
Ovocalyxin-32 (OCX32)	F: GGACAGCACTGCACTACATCAA	514	92
	R: GGAATTTCGTGGAGCAAGACAA		
Ovocalyxin-36 (OCX-36)	F: TTGGAATGGTCGTCTTCTGTGG	121	49
	R: CGGTCTGAATGATGGCATCG		
Ovocleidin-17 (OC-17)	F: CGTTCTGCCGCCGTTGGG	96	93
	R: CCCGCGACGCGTTGAGGA		
Ovocleidin-116 (OC-116)	F: AAGAGCCAACATCCAAGTGGGTGAGAAT	424	94
	R: CAGTGACCACATGGCTCCCTTTCCT		
Hepatic selenoproteins			
Glutathione peroxidase1	F: GCGACTTCCTGCAGCTCAACGA	99	10,11
	R: CGTTCTCCTGGTGCCCGAAT		
Glutathione peroxidase4	F: CGGTGAATTACACTCAGCTCGT	123	
	R: CTTTGATCTGCGCGTCGTCC		
Iodothyronine deiodinase1	F: AAGCTGCACCTGACCTTCATT	138	
	R: TTGTTTCTGAAGGCCCATCCA		
Iodothyronine deiodinase4	F: CAGTGTAATCCACATAGCCA	137	
	R: CTGAGCCAAAATTAACCACC		
Selenoprotein W1	F: CTCCGCGTCACCGTGCTCT	155	
	R: CTGCCCACCGTCACCTCGAAC		
Thioredoxin reductase1.	F: ACTGGATGACTATGACCGAA	103	
	R: TATGCATTCTCATACGTGAC		
Housekeeping			
Glyceraldehyde-3-phosphate dehydrogenase	F: AATGAGAGGTTCAGGTGCCC	150	10,11
	R: ACCAGACAGCACTGTGTTGG		
β-actin[8]	F: ACACACGGACACTTCAAGGG	128	

	R: TACTCAGCACCTGCATCTGC		
TATA-Box Binding Protein	F: TAGCCCGATGATGCCGTAT	147	95,96
	R: GTTCCCTGTGTCGCTTGC		

Figures

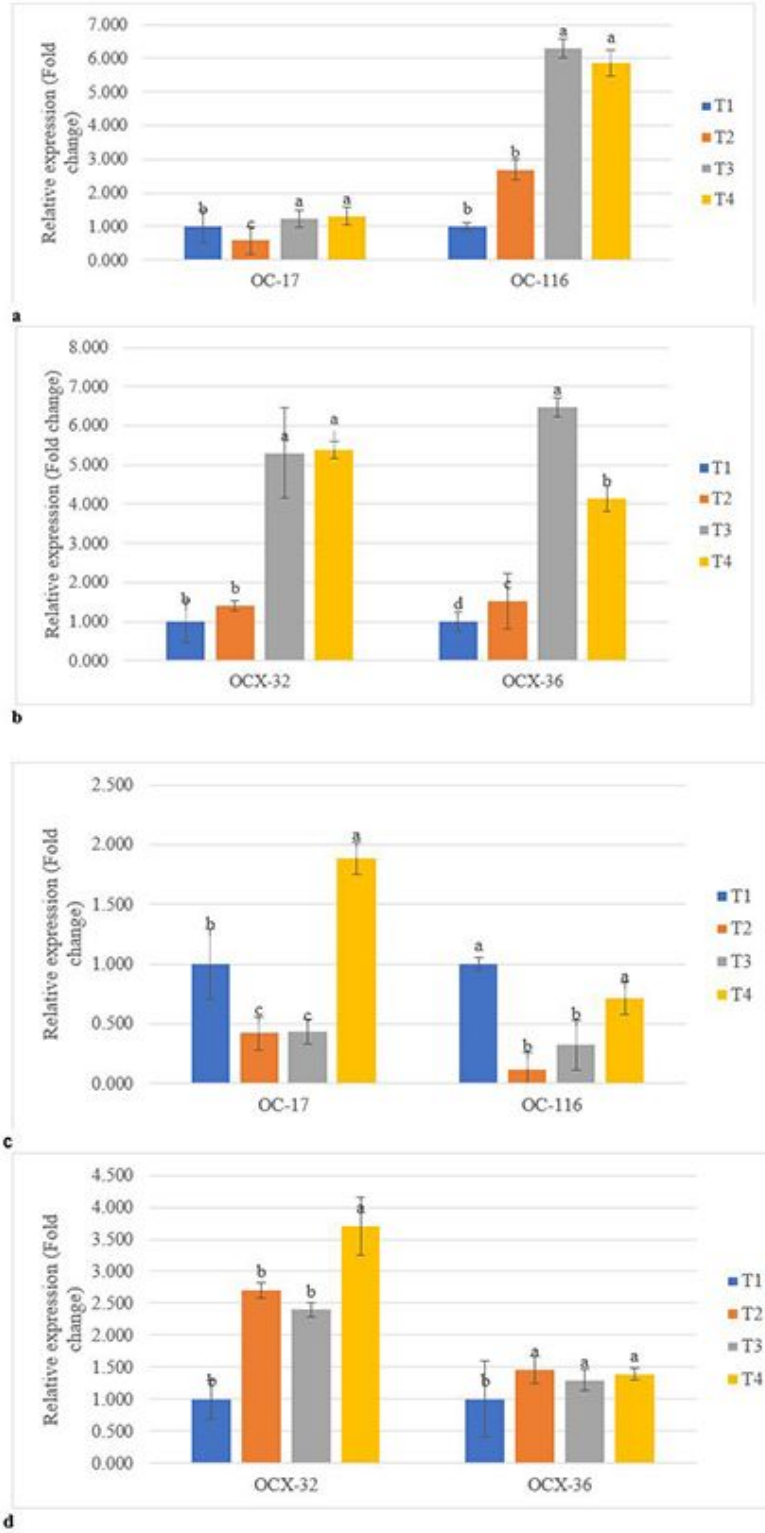
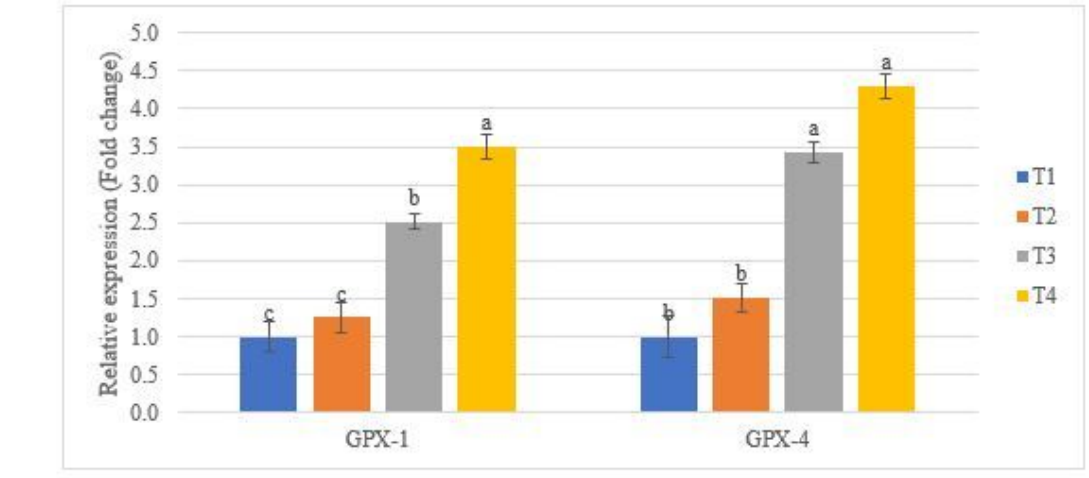
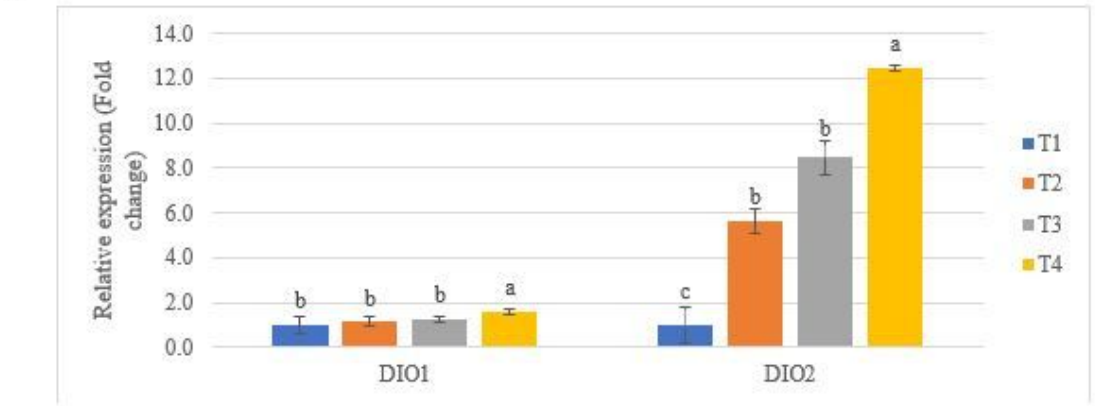


Figure 1

Relative expression levels of the calculated target genes affected by different dietary Se source in the uterus and magnum of laying hens. (a) OC-17 and OC-116 mRNA expression in the uterus. (b) OCX-32 and OCX-36 mRNA expression in the uterus. (c) OC-17 and OC-116 mRNA expression in the magnum. (d) OCX-32 and OCX-36 mRNA expression in the magnum. The fold changes were normalized with a housekeeping gene (GADPH and β-actin). Then, treated samples were expressed relative to the gene expression of the CON group (T1). Data represented as the means ± standard error. The x-axis represents hens' experimental groups, and Y-axis represents relative fold change for gene expression. Bars with different letters differ significantly at $P < 0.05$. Treatments: T1; basal diet, T2 basal diet + 0.3 mg/kg sodium selenite, T3: basal diet + 0.3 mg/kg Se-Yeast, T4: basal diet + 0.3 mg/kg Se of ADS18. Primer pairs used for these analyses are listed in Table 2.



a



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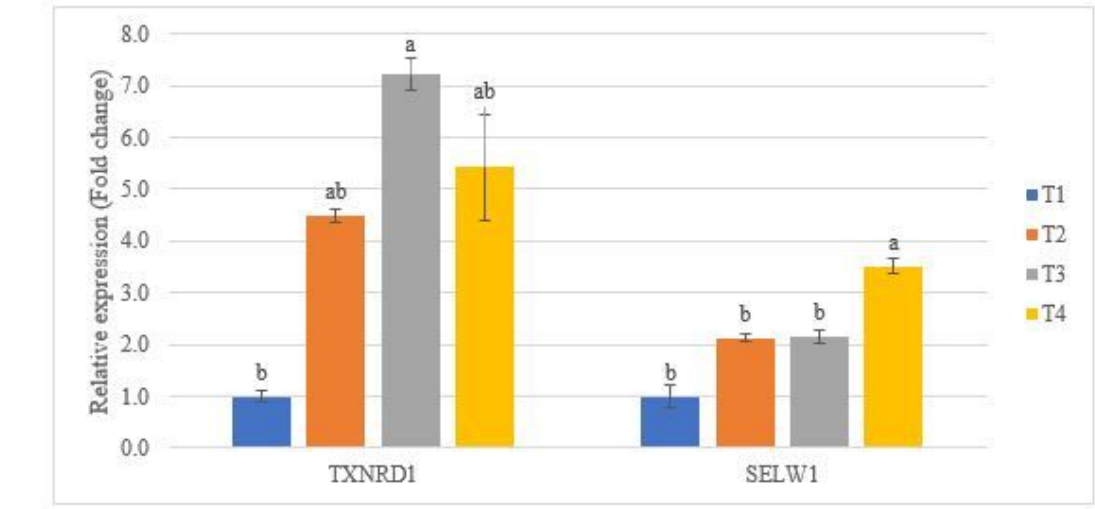

c

Figure 2

Relative expression of selenoprotein transcripts liver of laying hens fed different dietary Se source. (a) GSH-Px1 and GSH-Px4 mRNA expression. (b) DIO1 and DIO2 mRNA expression. (c) TXNRD1 and SELW1 mRNA expression. The fold changes were normalized with a housekeeping gene (GADPH and β-actin). Then, treated samples were expressed relative to the gene expression of the CON group (T1). Data represented as the means ± standard error. The x-axis represents hens' experimental groups, and Y-axis

represents relative fold change for gene expression. Bars with different letters differ significantly at $P < 0.05$. Treatments: T1; basal diet, T2 basal diet + 0.3 mg/kg sodium selenite, T3: basal diet + 0.3 mg/kg Se-Yeast, T4: basal diet + 0.3 mg/kg Se of ADS18. Primer pairs used for these analyses are listed in Table 2.