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# Selenium, thiobarbituric acid reactive substances, and thyroid hormone activation in broilers supplemented with selenium as selenized yeast or sodium selenite

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## Abstract

The objective of this experiment was to compare the efficiencies of sodium selenite (SS) and selenized yeast (SY) supplemented at different doses (0.05 and 0.30 mg Se/kg feed) with respect to plasma glutathione peroxidase (Gpx) activity, extent of oxidative lipid injury, and thyroid hormone activation in broilers during the first four weeks of growth. Results indicate a significant increase in plasma Gpx activity and reduction in thiobarbituric acid reactive substances (TBARS) in all supplemented groups at 4 weeks of age compared to 2-week-old chicks. Plasma thyronine activation was highest in SY supplemented broilers. It can be concluded that in the first 4 weeks of broiler life selenite has a more efficient antioxidative effect which is reflected in lower plasma and liver TBARS values. However, broiler feed supplementation with selenized yeast results in a more proficient conversion of T4 to T3.

Key words: Broilers, selenium, TBARS, thyroid hormones

## Introduction

The first connection between selenium (Se) and its antioxidative effect was determined by Rotruck *et al.*<sup>31)</sup> who described the enzyme glutathione peroxidase (Gpx; EC.1.11.1.9) present in rats' erythrocytes as a selenoenzyme. Further studies defined plasma Gpx as a tetrameric enzyme containing selenium in the form of selenocysteine. It has a protective antioxidant

role as it is capable of reducing highly toxic hydroperoxides into respective alcohols<sup>11,12)</sup>. The chain reactions of fatty acid auto oxidation, if not blocked by antioxidants, progress into the formation of free radicals, aldehydes, ketones and alcohols, all of which have toxic properties<sup>28)</sup>. The so formed malondialdehyde (MDA) easily reacts with 2-thiobarbituric acid giving a pink colored complex compound (thiobarbituric acid reactive substance; TBARS) which is a good

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indicator of the degree of lipid peroxidation.

The biochemical role of selenium is reflected not only in its antioxidative effect. Jensen *et al.*<sup>19)</sup> observed that dietary Se affects thyroid hormone metabolism. Plasma triiodothyronine (T3) is produced by 5'-deiodination of thyroxine (T4) in the thyroid, liver and kidney. This reaction is catalyzed by type I iodothyronine deiodinase (5'-ID)<sup>6)</sup>. In 1990 type I 5'-ID was verified to be a selenoenzyme by parallel studies of Arthur<sup>4)</sup> and Behne<sup>7)</sup>. It has been reported that Se deficiency affects both thyroid hormone synthesis in the thyroid gland and the activity of tissue specific 5'-ID in rats<sup>23)</sup>. It is documented that thyroid hormones play an important role in growth and protein turnover<sup>18,20)</sup>. Therefore, Se deficiency can affect the overall growth and productive performance in domestic animals by impairing T3 production.

Intensive growth burdens cell metabolism in broilers, thus increasing the need for essential nutrients such as Se. The Se requirement for broilers throughout the growth period is 0.15 mg/kg<sup>26)</sup>, and this requirement in many areas of the world can be seldom met by natural feedstuffs in the diet<sup>10,14,21,29)</sup>. Intake of Se-deficient feed can result in serious Se deficiency with corresponding health problems especially in highly productive animals such as poultry. Hence, it is common practice to supplement broiler diets with Se. The maximum allowable level of Se supplementation is 0.30 mg/kg<sup>1)</sup>. The form of supplemented Se that has been primarily used is the inorganic one, i.e. sodium selenite (Na<sub>2</sub>SeO<sub>3</sub>). However, due to safety reasons the interest to use organic forms of Se, such as Se-cysteine, Se-methionine, or Se enriched yeast, as supplemental sources of Se is increasing.

The objective of this experiment was to compare the efficiencies of sodium selenite (SS) and selenized yeast (SY) supplemented at different doses (0.05 and 0.30 mg Se/kg feed) in respect to plasma Gpx activity, thyroid hormone activation, and extent of oxidative lipid injury in broilers during the first four weeks of growth as

this period is the most demanding due to intensive growth and feathering.

## Materials and Methods

*Experimental animals:* The experiment was carried out on Arbor Acres broiler chicken from day 1 post hatching until 4 weeks of age. Chicks were housed and kept under standard conditions for commercial farms. Thirty chicks were sacrificed day 1 post hatching, the remaining 50 were randomly assigned to 5 experimental pens (10 birds each). The allotted broilers were supplemented with dietary Se in the form of SS or SY (Sel-Plex<sup>®</sup>, Alltech) at 0.05 or 0.30 mg Se/kg feed for the 4 week experiment. All experimental groups were set as replicates. The control group was unsupplemented. The concentration of naturally occurring Se in the basal diet was 0.088 mg/kg feed.

In order to limit the variable factors that may affect and further stress the animals they were fed a single corn and soybean meal feeding regimen throughout the trial. All nutrients met the nutrient requirements for broilers<sup>26)</sup> with the exception of Se which was supplemented additionally according to the experimental protocol.

The experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Belgrade.

*Blood samples:* On day 1 post hatching a total of 30 chicks were sacrificed as blood samples were taken by cardiac puncture. Samples were immediately pooled into 10 separate pools (n = 10, one pool containing equal quantities of blood from 3 chicks). Further blood sampling was carried out by venepuncture of *v. subclavia* at 2 and 4 weeks of age. Heparinized blood samples were centrifuged at 1500 × g for 20 min and plasma was separated. Determination of Gpx was carried out on fresh plasma samples. The remaining plasma was packed into 1.5 ml tubes and stored frozen for 2 weeks at -20°C until

subsequent TBARS, T4 and T3 analyses.

*Liver samples:* The control and supplemented groups were sacrificed at 4 weeks of age by decapitation under light ether anesthesia. The livers were removed, cleaned of blood, gall bladders and bile and homogenized. In fresh, refrigerated (4°C) liver samples the concentration of MDA was determined within 24 hr.

*Plasma glutathione peroxidase activity:* Glutathione peroxidase (Gpx) activity was measured by the coupled test<sup>15</sup>. The Gpx present in the plasma sample (5 µl) reduces *tert*-butyl hydroperoxide (TBH). Glutathione (GSH: 200 µl, final concentration 6 mM) as the donor of hydrogen becomes oxidized (GS-SG). In the second phase of this coupled reaction GS-SG is reduced to GSH by NADPH (200 µl; 0.30 mM) and glutathione reductase (EC.1.6.4.2, 50 µl, 0.375 IU/ml). Two minutes after TBH addition (500 µl, 1.575 mM) absorbance (A) readings at 366 nm were taken at 60 sec intervals during 3 min on a Cecil Ce2021 spectrophotometer with a Peltier thermostat unit. Low concentration of TBH (under 2.32 mM) as used in this method, determines only the activity of Se-dependent Gpx<sup>9</sup>. The optimal pH was provided by 500 µl phosphate buffer. The results were expressed in microkatal per liter (µkat/l), one katal being SI unit equal to the conversion rate of 1 mol of substrate per second. The former enzyme unit (IU) equals 16.67 nkat. All chemicals required for plasma Gpx activity measurement and the following plasma and liver TBARS determination were obtained from Sigma Aldrich.

*Plasma TBARS determination:* Plasma MDA concentration, expressed as thiobarbituric acid reactive substances (TBARS), was determined by the method described by Andreeva *et al*<sup>3</sup>. The advantage of this method is that it can be used not only with fresh but on previously frozen plasma samples, as well. To plasma samples (300 µl), 3.0 ml of 1% orthophosphoric acid, 1.0 ml

0.6% 2-thiobarbituric acid and 100 µl FeSO<sub>4</sub> (1 µmol) were subsequently added. After thorough mixing the stoppered conical glass tubes were set in a boiling water bath for 60 min. Tubes were cooled at room temperature and 4.0 ml n-butanol was added, mixed on a vortex and centrifuged at 2200 × g for 10 min. The separated butanol layer was then carefully transferred into cuvettes and absorbance was recorded at 535 nm. The blank consisted of n-butanol only.

*Liver TBARS determination:* The procedure was described by Uchiyama and Mihara<sup>39</sup>. To 50 ml distilled water 10 g of liver tissue was added and homogenized. Prior to homogenization 30 mg of the antioxidant *tert*-butylhydroxytoluene (BHT, Sigma Aldrich) were added. The homogenate was then transferred into a conical flask and 47.5 ml of distilled water and 2.5 ml 3.99 M HCl (pH 1.5) were added. Distillation was carried out in a sand bath until 50 ml of clear distillate was obtained. Out of the distilled volume 5 ml were taken and added to 5 ml 0.02 M 2-thiobarbituric acid and heated in a stoppered glass tube in a boiling water bath for 35 min. After cooling the absorbance was measured at 535 nm.

*Plasma T3 and T4 determination:* Plasma thyroxine and thyronine (T4 and T3) concentrations were determined in duplicate samples using standard commercial RIA kits (Institute for the Application of Nuclear Energy INEP, Zemun, Serbia). The intra- and inter-assay coefficients of variation were less than 10%.

*Statistical analysis:* Data are presented as means ± SD. The selection of birds from each treatment group, at each sampling time of the study was randomized. The data were analyzed using GraphPad Prism v.5 statistical software. The influences and interactions of treatments were analyzed using 2-way ANOVA, followed by Bonferroni post-hoc test. Student's *t*-test was utilized to determine the differences in plasma thyroid hormones concentrations and activation

ratios. In all cases a probability level  $p < 0.05$  was considered statistically significant.

## Results

Plasma Gpx activity levels are summarized in Table 1. Two-way ANOVA analysis reveals significant influences of both Se source ( $F = 3.766$ ,  $p < 0.01$ ) and age ( $F = 25.06$ ,  $p < 0.001$ ) on plasma Gpx activity of chicken, as well as a significant interaction between treatments ( $F = 2.462$ ,  $p = 0.0160$ ). The activity of Gpx in 1-day-old chicks was  $31.18 \pm 7.98 \mu\text{kat/l}$ . In chicks at 2 weeks of age there was no significant difference between the supplemented groups and the control. No significant difference in Gpx activity was detected between same doses of SY and SS. At 4 weeks there was an evident overall increase in plasma Gpx activity compared to

week 2. All Se supplemented groups except SS 0.05 had significantly higher plasma Gpx activities compared to Control. Group SY 0.05 had significantly higher Gpx activity ( $p < 0.01$ ) in comparison to SS 0.05.

Plasma TBARS levels are summarized in Table 2. There were significant influences of both Se source ( $F = 3.31$ ,  $p < 0.0001$ ) and age ( $F = 73.58$ ,  $p < 0.0001$ ) on plasma TBARS concentration in chicken, as well as a significant interaction between treatments ( $F = 5.26$ ,  $p < 0.0001$ ). At day 1 the chicks average plasma TBARS content was  $6.38 \pm 0.90 (\mu\text{M})$ . At 2 weeks these values sharply decreased. At 4 weeks of age plasma TBARS concentrations reverted to values close to those found in 1-day-old chicks. All supplemented groups had significantly lower plasma TBARS values compared to the control (Fig. 1A). Group SS 0.30 had significantly lower ( $p < 0.05$ ) plasma TBARS content compared to SS 0.05. Liver

**Table 1. Plasma Gpx activity in broilers 1 day, 2 weeks and 4 weeks old.**

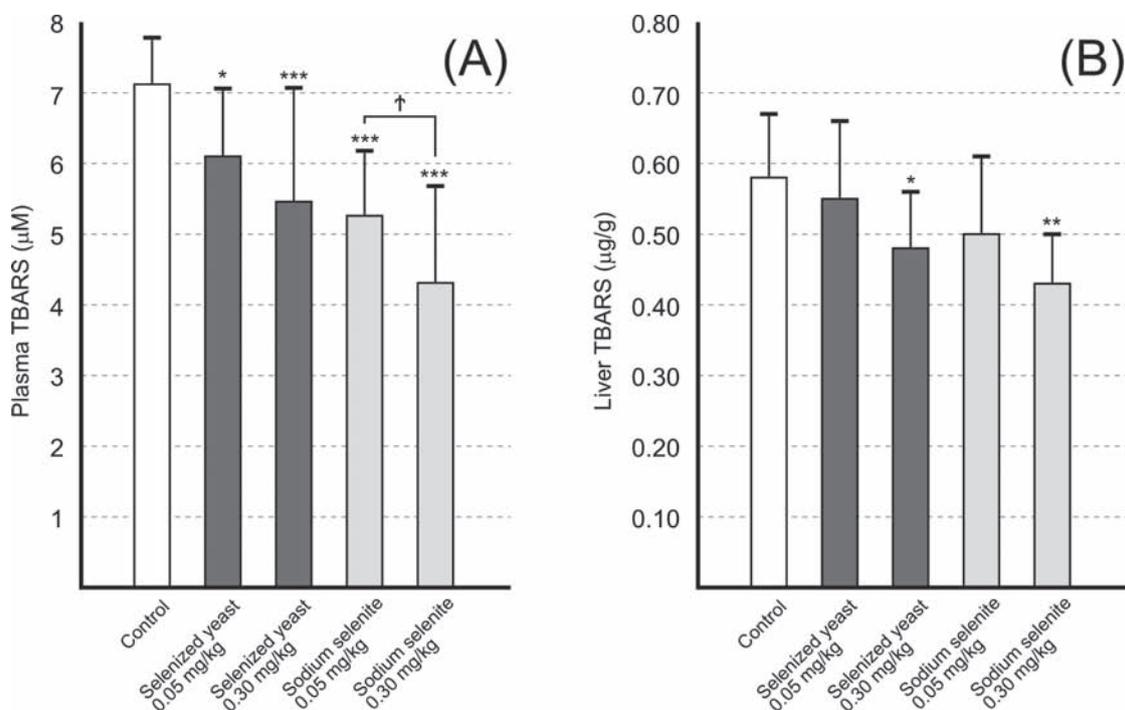
Group	Plasma Gpx activity ( $\mu\text{kat/l}$ )		
	1 day	2 weeks	4 weeks
Control	$31.18 \pm 7.98$	$21.46 \pm 2.24$	$27.94 \pm 4.99$
SY 0.05		$27.07 \pm 6.28$	$41.58 \pm 9.62^{\text{a,b}}$
SY 0.30		$26.06 \pm 9.46$	$41.41 \pm 9.67^{\text{a}}$
SS 0.05		$28.78 \pm 9.58$	$31.31 \pm 4.44^{\text{b,c}}$
SS 0.30		$26.09 \pm 5.09$	$41.58 \pm 9.16^{\text{a,c}}$

Groups were labeled according to the selenium supplementation source (SY: selenized yeast and SS: sodium selenite) and dose (0.05 and 0.30 mg Se per kg feed). <sup>a</sup> $p < 0.001$ , compared to Control; <sup>b-b,c,c</sup> $p < 0.01$  between groups

**Table 2. Plasma TBARS concentrations in broilers 1 day, 2 weeks and 4 weeks old.**

Group	Plasma TBARS ( $\mu\text{M}$ )		
	1 day	2 weeks	4 weeks
Control	$6.38 \pm 0.90$	$2.94 \pm 0.34$	$7.12 \pm 0.66$
SY 0.05		$2.02 \pm 0.28$	$6.10 \pm 0.96^{\text{a}}$
SY 0.30		$2.23 \pm 0.49$	$5.46 \pm 1.61^{\text{b}}$
SS 0.05		$2.83 \pm 0.47$	$5.26 \pm 0.92^{\text{b,c}}$
SS 0.30		$2.47 \pm 0.32$	$4.31 \pm 1.37^{\text{b,c}}$

Groups were labeled according to the selenium supplementation source (SY - selenized yeast and SS - sodium selenite) and dose (0.05 and 0.30 mg Se per kg feed). <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.001$  compared to Control; <sup>c-c</sup> $p < 0.05$  between groups



**Fig. 1. TBARS concentrations in 4-week old broilers in plasma (A) and liver (B).** Astrix (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ) and dagger (†,  $p < 0.05$ ) signs represent the significance of difference of supplemented groups compared to control or between supplemented groups, respectively. The effect of reduction of TBARS concentrations shows dependency on selenium source and dose.

**Table 3. Plasma T<sub>3</sub> and T<sub>4</sub> concentrations in 4 weeks old broilers.**

Group	Plasma T <sub>3</sub> (nM)	Plasma T <sub>4</sub> (nM)	Activation ratio (T <sub>3</sub> /T <sub>4</sub> × 100)
Control	2.97 ± 0.47	79.0 ± 7.9	3.78 ± 0.64
SY 0.05	3.53 ± 1.02 <sup>a</sup>	81.3 ± 15.5	4.57 ± 0.46 <sup>a</sup>
SY 0.30	3.43 ± 0.85 <sup>a</sup>	70.0 ± 7.07 <sup>a</sup>	4.90 ± 1.08 <sup>b</sup>
SS 0.05	3.24 ± 0.80 <sup>a</sup>	84.3 ± 14.4 <sup>a</sup>	3.87 ± 0.66
SS 0.30	3.38 ± 0.57 <sup>a</sup>	86.7 ± 12.4 <sup>a</sup>	4.01 ± 0.23

Groups were labeled according to the selenium supplementation source (SY - selenized yeast and SS - sodium selenite) and dose (0.05 and 0.30 mg Se per kg feed). Activation ratios were calculated for individual chicks prior to statistical analysis. <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$  compared to Control

TBARS concentrations in 4-week-old broilers (Fig. 1B) followed the pattern similar to plasma.

Although plasma T<sub>4</sub> levels variations were apparently independent of Se source, plasma T<sub>3</sub> values (Table 3) were markedly higher in all Se supplemented groups compared to the control. The ratio of plasmatic T<sub>3</sub>, which is the active form of the hormone, relative to T<sub>4</sub> (the inactive prohormone) was higher in groups supplemented with selenized yeast at both given doses.

## Discussion

Blood plasma Gpx activity can be used as a reliable indicator of the functional Se status in man and animals. This relationship is best described by a logarithmic equation. It is considered that the plateau of maximal Gpx activity in pigs is reached at 0.10 mg/kg dietary Se<sup>25</sup>. Earlier studies<sup>13</sup>) indicate that increasing Se supplementation up to 0.2 mg/kg results in a

steady increase in Gpx activity. After this point a steady activity plateau is maintained. These results are in agreement with our findings, as our results have established that throughout the 2-week trial plasma Gpx activity was not affected neither by Se level of supplementation nor by source of supplemented dietary Se. This is probably due to the fact that within one week after the birth the antioxidative status of chicks depends on the Se and vitamin E accumulated in the liver during embryogenesis, and does not depend on the feed Se content<sup>36)</sup>. However, the increased plasma Gpx activity in 4-week-old broilers compared with 2-weeks-old chicks is in agreement with the results published by Kuricova *et al.*<sup>24)</sup> who described a steady Gpx activity increase in broilers from week 2 to week 4 of age in birds supplemented with 0.20 mg/kg SS and 0.20 mg/kg SY. Payne and Southern<sup>30)</sup> fed broilers SS or SY at 0.30 mg/kg for a 49 day trial and reported no statistical difference between treatments. This is in agreement with our results obtained at 4 weeks. However, at this stage the group fed SS 0.05 mg/kg plasma Gpx activity was lagging behind, indicating that such supplement was unable to secure the plateau of enzyme activity.

Based on the known metabolic pathways for Se<sup>36)</sup> we would not expect a marked increase in Gpx activity from an organic Se source such is SY which is known for its high content of selenomethionine<sup>8)</sup>. This is because Se has to be converted into selenocysteine before it can be incorporated into Gpx<sup>12)</sup>. It was reported<sup>35)</sup> that inorganic Se from selenite was efficiently metabolized into selenocysteine, whereas on a later date the low rate of conversion of selenomethionine into selenocysteine was described<sup>18)</sup>. The determination of MDA concentration in tissues, which is expressed as plasma TBARS, represents one of the major indicators of fatty acids peroxidation. The obtained results clearly indicate that the degree of fatty acid peroxidation and the subsequent plasma and tissue MDA concentration change

during broiler growth and depend upon the presence of antioxidants, such as Se supplemented to the feed. At day 1 plasma TBARS were significantly higher compared to 2 weeks of age. This can be explained by the high oxidative stress to which chicks are exposed immediately after hatching<sup>38)</sup>. The highest plasma TBARS value measured at 2 weeks was in the unsupplemented group ( $2.94 \pm 0.34 \mu\text{M}$ ). All treated groups had significantly lower TBARS values compared to the control. Similar findings were reported by Guo *et al.*<sup>16)</sup> and Actis-Gorretta *et al.*<sup>2)</sup>. At 4 weeks of age there was a twofold increase in plasma TBARS values. It is interesting to note that at this time plasma TBARS levels show no significant differences between equivalent doses of SS and SY, but the antioxidative effect is dose dependent (Fig. 1).

A number of studies have established that feed supplementation with a combination of two antioxidants, such as Se and vitamin E, ensures an additional protective effect resulting in significantly lower TBARS in plasma, liver and muscle tissue<sup>27,32,33)</sup>.

The effects of Se on liver TBARS in the current investigation retained a similar pattern to the changes observed in the plasma at the same time of sampling (4 weeks). Groups supplemented 0.30 mg Se per kg feed either as SY or SS had significantly lower liver TBARS content compared to control, but no significant differences could be found between groups. These observations partially contradict the description of a significant ( $p = 0.001$ ) decrease in liver TBARS in quails supplemented with 0.20 mg/kg SS compared to 0.10 mg/kg SS<sup>34)</sup>.

Soon after the observation<sup>19)</sup> that dietary Se affects thyroid hormone metabolism Beckett *et al.*<sup>5)</sup> found that T3 is produced by 5'-deiodination of T4 particularly in the thyroid, liver and kidney. The activity of the selenoenzyme catalyzing 5'-deiodination in rats is affected by Se deficiency<sup>23)</sup>. It has been reported that hepatic 5'-ID activity in the Se deficient rat is 10-fold lower than that of the Se supplemented rat and

plasma T3 concentration is significantly lower than that of the normal rat<sup>6)</sup> which is in accordance with our results.

In all Se supplemented birds plasma T4 was apparently independent from Se source, while T3 concentration was significantly higher compared to the untreated control group. However, no significant differences were recorded between the treated groups (Table 3). The distinction of the efficacy of T4 to T3 conversion was observed only after comparison of the concentration ratio between the hormone and prohormone ( $T3/T4 \times 100$ ) which strongly suggested that organic Se supplementation facilitated the conversion of T4 to T3. The increased effectiveness in the SY supplemented groups was a consequence of a moderately to significantly lower plasma T4 concentration compared to SS supplemented groups. This is probably the result of the negative feedback control mechanism through the thyroid axis, which under normal conditions inhibits an excessive increase of plasma T3. A comparative effect was also noted in our studies of other animal species such as calves and heifers<sup>22)</sup>. The implications of this mechanism could be relevant in mildly hypothyroid individuals in which a diet fortified with easily available Se, such as selenized yeast, could, at least for some time, increase the conversion of T4 to T3 without a simultaneous increase of T4 release from the thyroid.

In our experiment Se supplementation provided Se concentrations in the feed above the level of Se deficiency. The percentual contribution of plasma T3 (active hormone) relative to the prohormone T4 was higher in groups receiving SY compared to SS. However, there was no marked dose dependence within treatments.

From the above results it can be concluded that within the first 4 weeks of broiler life the antioxidative effects of selenite and selenized yeast appear to be similar which is reflected in lower plasma and liver TBARS concentrations in comparison to controls; however feeding broilers

with the supplement containing selenized yeast results in a more proficient conversion of T4 to T3.

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