https://doi.org/10.17221/148/2018-CJAS

Effects of dietary manganese on antioxidant status, biochemical parameters and thickness of intestinal mucus in laying hens

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Citation: Piešová E., Faixová Z., Maková Z., Venglovská K., Grešáková Ľ., Faix Š., Čobanová K. (2019): Effects of dietary manganese on antioxidant status, biochemical parameters and thickness of intestinal mucus in laying hens. Czech J. Anim. Sci., 64: 99–106.

Abstract: The objective of this study was to investigate the effects of different sources of manganese (Mn) on the antioxidant status, some biochemical parameters and thickness of intestinal mucus in laying hens. Ninety-six 20-week-old hens of the Lohman Brown strain were randomly divided into four groups; each group was replicated six times with four hens per replicate. Hens in the control group were fed the basal diet (BD) with natural background Mn level of 45.5 mg/kg feed. For the three experimental groups, the BD was supplemented with 120 mg Mn/kg either from Mn-sulphate or Mn-chelate of protein hydrolysate (Mn-Bioplex) or Mn-chelate of glycine hydrate (Mn-Glycinoplex). After eight weeks of dietary treatment, a tendency towards increasing activity of superoxide dismutase (SOD) in the erythrocytes was observed in all experimental groups. The results showed that inorganic Mn source increased the glutathione peroxidase activity in the hens' blood ($P < 0.05$). The intake of Mn-Bioplex resulted in significantly increased total antioxidant status in the plasma of hens in the group ($P < 0.05$) compared to the control group. The plasma biological antioxidant potential was increased in the treatment groups supplemented with Mn-sulphate ($P < 0.01$) and Mn-Bioplex ($P < 0.05$). The total cholesterol concentration was decreased ($P < 0.05$) in the group fed the diet supplemented with Mn-Glycinoplex, as compared with the control. Moreover, the mucus layer thickness in the jejunum was reduced in the groups of birds fed with the organic form of Mn ($P < 0.001$, $P < 0.05$), as well as with the inorganic form of Mn ($P < 0.001$). These results indicate that the addition of dietary Mn was effective in improving the biological antioxidant potential, decreasing total plasma cholesterol and reducing the thickness of adherent intestinal mucus gel in the jejunum of laying hens.

Keywords: layers; manganese chelates; antioxidant enzymes; production of mucus

Poultry diets require supplementation with manganese (Mn) because of the relatively low availability of Mn from maize-soybean meal-based diets. Mn requirements for laying hens have been shown to vary considerably from 30 to 200 mg/kg depending on the criteria of response, production cycle and age of hens (Attia et al. 2010). Biological availability from different sources of Mn and its supplementation has been studied by many authors (e.g. Baker and Halpin 1987; Fly et al.

Supported by the Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic and the Slovak Academy of Sciences (VEGA 1/0658/17 and 2/0069/17).

https://doi.org/10.17221/148/2018-CJAS

1989). In commercial poultry diets Mn is supplemented in the form of inorganic salts such as sulphates, oxides and carbonates, or in organically complexed forms of Mn such as chelates or proteinates. Several studies have shown that organic complexes result in higher bioavailability of Mn than inorganic salt analogues (e.g. Leeson 2003). The supplementation of Mn provides such level of the mineral which prevents manifestation of clinical deficiency and allows the birds to achieve their genetic growth potential (Bao et al. 2007). Mn is an essential micronutrient which serves as a co-factor for many enzyme systems. Metalloenzymes such as arginase, pyruvate carboxylase and manganese superoxide dismutase (Mn-SOD) require Mn for their function (Burlet and Jain 2013). Mn-SOD is the major mitochondrial antioxidant and is responsible for protecting cells from reactive oxygen species (ROS) by scavenging mitochondrial superoxide (Fridovich 1997). Mn-SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide, which can be reduced to water by other antioxidant enzymes (Leach and Harris 1997). The crucial role of Mn-SOD in protecting cells against oxidative stress has been extensively studied (Sarsour et al. 2012). An oxidative stress condition is created in cells as a result of an imbalance between the generation and the detoxification of ROS. Several studies have reported that changes in dietary Mn induced changes in Mn-SOD activity (e.g. Davis et al. 1990). Other studies have also shown the beneficial effect of Mn on lipid peroxidation and a decrease in total serum cholesterol (Bomb et al. 1988).

The absorption of Mn takes place in the small intestine. Intestinal absorption of Mn occurs through a divalent metal transporter 1 (DMT1)-mediated active transport, as well as by passive diffusion. The export of Mn from the enterocyte may involve pathways of iron export and binding to transferrin (Kiela and Ghishan 2016). The intestinal tract epithelium is covered by a mucus layer composed predominantly of mucin glycoproteins, which are synthesized and secreted by the goblet cells. This layer acts as a barrier between the luminal contents and the absorptive system of the intestine. Changes in the properties of this barrier could affect the absorption of both dietary and endogenous macromolecules and ions (Quarterman 1987; Satchithanandam et al. 1990). Mn absorption may be influenced by several dietary factors including calcium (Davidsson et al. 1991), potassium (Wedekind et al. 1991) and nickel (Finley and Monroe 1997), and the sources of dietary carbohydrates and proteins (Keen et al. 1986), but the greatest effect on Mn absorption comes from dietary iron (Rossander-Hulten et al. 1991). The outcome of a study of Ibrahim et al. (2016) showed that pretreatment with Mn (II) complex before acidified ethanol administration was able to increase the quantity of stomach mucus production in rats.

The aim of our study was to determine the effects of inorganic and organic sources of Mn on antioxidant status, selected biochemical parameters and thickness of the mucus adherent layer in the small intestines of laying hens.

MATERIAL AND METHODS

All procedures during the experiments were in accordance with European Community guidelines (Directive 2010/63/EU) for animal experiments, and the experimental protocol was approved by the Ethics Committee of the Institute of Animal Physiology of the Slovak Academy of Sciences, and the State Veterinary and Food Administration (Ro-1479/11-221/3).

Animals, diets, and management. A total of ninety-six 20-week-old hens of the Lohman Brown laying strain were assigned to four dietary treatment groups based on their body weight. Each group was replicated six times with four hens (two cages) per replicate. The cages with size of 43 × 42 × 68.5 cm provided a floor area of 903 cm^2 per hen. During the whole experiment the birds were fed a restricted amount of feed (120 g/day). Water was available *ad libitum*. The environmental temperature was kept at 19–24°C and relative air moisture at 60–70%. The daily photoperiod consisted of 15 h of light and 9 h of darkness. All the hens were fed the same wheat-maize-soybean meal basal diet formulated to contain adequate levels of all nutrients as recommended by the National Research Council (1994). The birds in the control group were fed the basal diet (BD) unsupplemented with Mn during the whole experiment, while those in the treatment groups were fed the control diet supplemented with either $MnSO_4 \cdot H_2O$ (laboratory grade), Mn-chelate of protein hydrolyzate (Bioplex® Mn 15%; Alltech Inc., USA) or Mn-chelate of glycine hydrate (Glycinoplex Mn 22%; Phytobiotics Futterzusatzstoffe GmbH, Germany) at 120 mg Mn per kg.

https://doi.org/10.17221/148/2018-CJAS

Table 1. Ingredients and calculated composition of the basal diet

Ingredients	Composition
Wheat, ground (%)	33.5
Maize, ground (%)	31.0
Soybean meal, extracted (%)	24.5
Limestone (%)	9.0
Premix HYD-10[1] (%)	2.0
Dry matter (g/kg)	888.3
Crude protein (g/kg)	176.0
Crude fibre (g/kg)	28.8
Metabolizable energy (MJ)	11.6
Methionine (g/kg)	3.8
Methionine + cystine (g/kg)	6.9
Lysine (g/kg)	8.7
Calcium (g/kg)	37.4
Phosphorus (g/kg)	5.9
Zinc (mg/kg)	67.5
Copper (mg/kg)	14.9
Manganese (mg/kg)[2]	45.5

[1]vitamin-mineral premix provided per kg of complete diet: vitamin A 11 000 IU, vitamin D_3 2 750 IU, vitamin K 2.2 mg, vitamin E 12.0 mg, vitamin B_1 2.2 mg, vitamin B_2 5.0 mg, vitamin B_6 3.1 mg, vitamin B_{12} 0.02 mg, niacin 24.6 mg, pantothenic acid 6.6 mg, biotin 0.1 mg, folic acid 0.6 mg, methionine 1.2 g, Ca 3.4 g, P 2.27 g, Cl 2.1 g, K 5.2 mg, Na 1.4 g, Zn 37.6 mg, I 0.4 mg, Co 0.2 mg, Cu 7.6 mg, Fe 48.1 mg, Se 0.1 mg, Mg 11.4 mg

[2]analysed value

The experimental period lasted for eight weeks. Mn content in the control diet was 45.5 ± 5.6 mg Mn/kg by analysis. The composition and nutrient content of the diet are given in Table 1. The mean analysed values of total Mn concentrations in the supplemented diets (three replicates of each) were (in mg/kg) 164.8 ± 4.3 ($MnSO_4 \cdot H_2O$ treatment), 172.9 ± 10.3 (Mn-Bioplex treatment), and 172.4 ± 4.5 (Mn-Glycinoplex treatment), respectively.

Sample collections and measurements. At the end of the experiment, one bird from each replicate was slaughtered for sample collection (six birds per treatment). Blood samples were collected into the heparinised tubes and centrifuged at 1180 *g* for 10 min, and plasma was removed for analysis. Tissue samples from the livers were immediately flushed with ice-cold saline and stored at –70°C until analysis. For measurement of the thickness of intestinal mucus, samples of duodenum, jejunum and ileum were collected from the identical intestine segment of each laying hen.

Chemical analyses. The activities of superoxide dismutase (SOD, EC 1.15.1.1) in the erythrocytes and glutathione peroxidase (GPx, EC 1.11.1.9) in the blood, total antioxidant status (TAS) in the plasma and content of haemoglobin (Hb) in the blood were analysed using commercial kits (Randox, UK). The activity of GPx in the liver was measured spectrophotometrically by monitoring oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm, as described by Paglia and Valentine (1967). The protein concentration in the liver was determined using the spectrophotometric method published by Bradford (1976). The malondialdehyde (MDA) concentration in plasma and liver was measured fluorometrically as shown by Jo and Ahn (1998).

Measurements of reactive oxygen metabolite (ROM) levels and biological antioxidant potential (BAP) were conducted using commercial kits (Diacron, Italy) and a FRAS4 device (H&D, Italy) according to the manufacturer's instructions. The principle of the d-ROMs test is to measure the concentration of hydroperoxides which are present in the sample using the Fenton reaction. The results of this test are expressed as CARR units (CARR U). One CARR U is equivalent to 0.08 mg/dl of hydrogen peroxide. The BAP test is based on the ability of a coloured solution which contains ferric ions bound to a chromogenic substrate (a thiocyanate derived compound) to decolour upon reduction of ferric to ferrous ions. The results of the BAP test are expressed in μmol/l of reduced iron.

The selected biochemical parameters were determined using a Genesys 10UV spectrophotometer analyser (Rochester, USA). The commercial kits (Randox, UK) were used for assigned total proteins and cholesterol; for assigned albumin and glucose we used kits from Erba Lachema s.r.o., Czech Republic.

Measurement of mucus adherent layer thickness. Segments of the duodenum, jejunum and ileum were processed for production of mucus, applying the method of Smirnov et al. (2004) and Thompson and Applegate (2006). The optical density of samples was measured with ELISA reader (Chromspec Microplate Reader, Slovakia) at 630 nm wavelength. The amount of adherent mucus was calculated by means of a standard

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Table 2. Effect of diet supplemented with different Mn sources on selected antioxidant parameters in laying hens

	Control (BD)	BD + $MnSO_4 \cdot H_2O$	BD + Mn-Bioplex	BD + Mn-Glycinoplex	SEM	*P*-value
SOD blood (U/g Hb)	1033.62	1221.73	1160.85	1100.19	29.20	0.11
GPx blood (U/g Hb)	80.12^{a}	100.50^{b}	84.74^{ab}	88.56^{ab}	2.55	0.02
GPx liver (U/g Hb)	20.22	19.06	19.13	19.71	0.23	0.25
MDA plasma (μmol/l)	0.34	0.32	0.39	0.36	0.01	0.31
MDA liver (nmol/g protein)	51.15	52.91	57.59	53.44	1.69	0.61
TAS plasma (nmol/l)	0.99^{a}	1.24^{ab}	1.36^{b}	1.20^{ab}	0.05	0.06
dROMs plasma (CARR U)	32.60	27.00	32.60	27.80	1.89	0.63
BAP plasma (μmol/l)	1694.60^{a}	2451.60^{b}	2311.00^{b}	2110.00^{ab}	88.88	0.001

BD = basal diet, SOD = superoxide dismutase, Hb = haemoglobin, GPx = glutathione peroxidase, MDA = malondialdehyde, TAS = total antioxidant status, dROMs = dROMs test, BAP = biological antioxidant potential test

^{a,b}means within a row with different superscripts significantly differ; $n = 6$

curve and expressed as μg of Alcian blue (AB) (AppliChem GmbH, Germany) per cm^2 of intestine.

Statistical analysis. The differences between the treatment groups were tested using one-way analysis of variance (ANOVA) with post hoc Tukey's multiple comparison test using GraphPad Prism Software (Version 5.02, 2008). Differences between groups were considered statistically significant at $P < 0.05$. Values in tables are given as means and pooled standard errors of the mean (SEM).

RESULTS

The effects of dietary Mn supplementation relating to the sources used on the evaluation of the antioxidant status of laying hens are presented in Table 2. After the feeding of diets with one inorganic and two organic Mn sources, a tendency towards increased SOD activity was observed in the erythrocytes of the hens. However, no significant differences between treatment groups were noted ($P > 0.05$). The supplementation of inorganic Mn to the diet resulted in a significant increase in GPx activity in the hens' blood in comparison with the group fed with the basal diet ($P < 0.05$). GPx activity in the liver and concentrations of MDA in the plasma and liver were not affected by the supplementation of Mn sources. The addition of Mn-Bioplex to the hens' diets significantly increased the total antioxidant status in their plasma compared to the control group ($P < 0.05$). Oxidative stress was measured with a reactive oxygen metabolites (ROMs) test, and antioxidant potency was evaluated with a biological antioxidant potential (BAP) test in plasma. No differences in plasma ROMs levels were detected between the dietary groups ($P > 0.05$). Compared to the control group, the biological antioxidant potential was significantly increased in groups supplemented with inorganic Mn ($P < 0.01$) and Mn-Bioplex ($P < 0.05$) sources.

The results of selected biochemical parameters are summarised in Table 3. After eight weeks of feeding

Table 3. Effect of diet supplemented with different Mn sources on selected biochemical parameters in the plasma of laying hens

	Control (BD)	BD + $MnSO_4 \cdot H_2O$	BD + Mn-Bioplex	BD + Mn-Glycinoplex	SEM	*P*-value
Total proteins (g/l)	61.20	60.04	58.86	60.65	0.50	0.41
Albumin (g/l)	16.60	18.31	17.96	18.68	0.35	0.16
Cholesterol (mmol/l)	3.36^{a}	3.50^{a}	3.13^{ab}	2.29^{b}	0.14	0.003
Glucose (mmol/l)	13.83	14.57	13.33	14.86	0.27	0.18

BD = basal diet

^{a,b}means within a row with different superscripts significantly differ; $n = 6$

https://doi.org/10.17221/148/2018-CJAS

Table 4. Effect of diet supplemented with different Mn sources on the intestinal mucus layer thickness of laying hens (μg Alcian blue/cm^2 of intestine)

	Control (BD)	BD + $MnSO_4 \cdot H_2O$	BD + Mn-Bioplex	BD + Mn-Glycinoplex	SEM	*P*-value
Duodenum	4.04^{ab}	4.48^{a}	3.63^{b}	4.42^{ab}	0.12	0.04
Jejunum	4.38^{a}	3.15^{b}	3.24^{b}	3.58^{b}	0.13	< 0.001
Ileum	4.98	4.71	4.82	4.31	0.13	0.31

^{a,b}means within a row with different superscripts significantly differ; $n = 6$

birds with the organic Mn-Glycinoplex enriched diet, significantly lower cholesterol concentrations were found in their blood plasma than in the plasma of control group ($P < 0.05$). No significant responses were recorded in blood concentrations of total protein, albumin and glucose.

The effects of Mn supplementation on the thickness of the hens' small intestine mucus adherent layer are given in Table 4. The mucus layer was significantly reduced in the jejunum of hens in all groups fed with addition of Mn ($MnSO_4 \cdot H_2O$ and Mn-Bioplex ($P < 0.001$); Mn-Glycinoplex ($P < 0.05$)) compared to the group fed only the basal diet. A significant difference was detected between the groups fed diets supplemented with $MnSO_4 \cdot H_2O$ and Mn-Bioplex in the production of mucus in the duodenum ($P < 0.05$).

DISCUSSION

In this study we compared the effects of dietary supplementation of the same amount of Mn but in two different forms (inorganic and organic) on selected antioxidant and biochemical parameters and the thickness of the mucus adherent layer in laying hens. In broiler chickens and laying hens, the influence of Mn on productive performance, meat quality and egg quality has already been identified (Lu et al. 2006; Xiao et al. 2015), but studies related to dietary Mn supplementation with organically complex minerals on antioxidant status in laying hens are scarce. The antioxidant systems of the living cell are based on three major levels of defence, and superoxide dismutase (SOD) is known to belong among the first components of the antioxidant defence network. Mn-SOD is shown to be highly expressed in various organs containing a large number of mitochondria. It has been proven that Mn availability is a regulating factor of Mn-SOD activity. It seems likely that Mn-SOD activity is very sensitive to dietary Mn levels in commercial corn-soybean meal diets (Luo et al. 1992). In the study by Saripinar-Aksu et al. (2010) with broiler chickens (Ross 308), the effect of using organic complexes of minerals Cu, Zn and Mn at 1/3, 2/3 and 3/3 portions of inorganic forms of those minerals revealed that erythrocyte SOD activity increased in all groups supplemented with organic complex minerals in comparison to those fed with inorganic forms. For SOD activity, however, only numerical increase was detected in all of the supplements we tested. One of the important cell redox pathways is the glutathione (GSH) and GSH/GPx enzyme system. The study by Bulbul et al. (2008) was conducted to compare the effects of dietary supplementation of Mn-proteinate (5 mg/kg) and MnO (30 mg/kg) on plasma MDA, GSH and antioxidant activity (AOA) in laying hens. Both Mn sources decreased the plasma MDA concentration, but left the plasma GSH and AOA unchanged. Bulbul et al. (2008) concluded that the decreased MDA concentration might be due to the contribution of Mn to both the formation of Mn-SOD and the formation of Mn-porphirine. In our study we found a significant increase in GPx activity in the blood after supplementation with the inorganic Mn form, but GPx activity in the liver and concentrations of MDA in the plasma and liver were not affected. Similar results have been reported in laying ducks (Fouad et al. 2016). Although plasma concentrations of total SOD were enhanced, MDA did not decline in this study. On the other hand, in broiler chickens dietary Mn supplementation enhanced the activity of Mn-SOD and decreased MDA concentration (Lu et al. 2006, 2007). Evidently, the relationships between dietary Mn and the activities of antioxidant parameters in laying hens need to be further investigated. An assay for measurement of plasma oxidants (d-ROMs) was performed in our study. ROM concentration was recently indicated as a potent marker of oxidative injury (Samouilidou et al. 2007). Using the d-ROMs assay we found no significant increase in ROM levels during our ex-

https://doi.org/10.17221/148/2018-CJAS

periments. The TAS and BAP assays are intended for direct measurement of the total antioxidant activity in the sample. These assays are intended for measurement of various antioxidants which are present in the sample (Ruskovska et al. 2014), so they are not specific for a particular antioxidant. This could explain the non-significant increases in the other antioxidant enzymes tested in our experiment. Our results demonstrate that organic Mn supplementation (as Mn-Bioplex) significantly increases the total antioxidant status and biological antioxidant potential in the plasma of laying hens. In addition, biological antioxidant potential was also increased in the plasma of hens fed with Mn supplementation from Mn-sulphate. Manganese is involved in cholesterol metabolism. Manganese supplementation has been shown to cause a decrease in total serum cholesterol, aorta cholesterol, and regression of atherosclerosis in cholesterol-fed rabbits (Bomb et al. 1988). The results of the study by Bae et al. (2011) suggest that Mn supplementation (as $MnCO_3$) resulted in reduction of the blood cholesterol levels in Ca-deficient ovariectomized rats. The findings presented in our study demonstrate that dietary supplementations of Mn in organic forms significantly lower total serum cholesterol in hens. Saripinar-Aksu et al. (2010) also reported that total cholesterol levels were decreased in chickens fed with organic complex minerals. Mineral utilization by animals primarily depends on their absorption from the ingested feed. Secreted mucins and the thickness of the adherent mucus layer in the gut influence nutrient digestion and absorption processes (Smirnov et al. 2004). The optimal level of mucin synthesis and secretion is unclear, but it is clear that there is a critical balance between their synthesis and degradation. Excessive mucin secretion increases endogenous nutrient losses and impairs nutrient absorption (Jeurissen et al. 2002). On the other hand, by reducing the mucus layer, nutrient retention by the animal should be increased (Cowieson et al. 2004). Bai et al. (2008), who studied the kinetics of Mn absorption in ligated small intestinal segments of broilers, concluded that Mn absorption showed a saturable carrier-mediated process in the duodenum and jejunum, whereas a non-saturable diffusion process was present in the ileum. In the study by Ji et al. (2006), the effect of Mn source on Mn uptake was evaluated using the technique of *in vitro* everted gut sacs. The results from that study indicate that the ileum is the main site of Mn absorption for broilers, and the uptake of Mn from organic sources was higher than that from inorganic sources. Sahagian et al. (1966) analysed intact strips of rat intestine *in vitro* and found that Mn was absorbed predominantly in the duodenum, while Mn uptake was least in the ileum. Garcia-Aranda et al. (1983) reported that Mn was better absorbed in the jejunum than in the ileum of rats using an *in vivo* perfusion system. There are no published observations on the effect of manganese on production of mucus in the small intestine in hens. In our experiment, the thickness of the mucus adherent layer was most reduced in the jejunum after supplementation with all Mn sources. The lower amounts of adherent mucus in the gut may be explained by a higher rate of degradation of the mucus layer, a lower rate of mucus secretion or an altered rate of mucin turnover. Further studies are needed involving detection of the influence of various components of feed on the production of the mucus adherent layer in the small intestine of hens.

CONCLUSION

The results of this experiment demonstrate that different sources of Mn used as dietary supplements significantly increase the biological antioxidant potential of laying hens, which could be used for preventing the formation of ROS. Our study also suggests that supplementation of hens' diet with organic Mn sources may decrease their total cholesterol concentration. Additionally, this study also indicates a clear reduction in the mucus layer thickness in the jejunum of hens.

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Received: 2018–08–07
Accepted: 2018–11–12