Effects of nano-manganese on humoral immune response and oxidative stress in broilers

Sepideh Sabagh1, Jamshid Razmyar2, Mohammad Heidarpour1*

1 Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran; 2 Department of Avian Health and Diseases, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

Abstract

The objective of the present study was to evaluate the alterations in selected indicators of immune responses and oxidative stress of broilers fed with nano-manganese. One hundred-sixty 1-day-old broiler chicks were randomly assigned into four groups with three replicates. Birds were fed the same basal diet supplemented with nano-manganese oxide, as 0.00 (control group), 50.00, 100, or 150 mg kg⁻¹ of diet. The birds were vaccinated against avian influenza (AI), Newcastle disease (ND), infectious bronchitis (IB) and infectious bursal disease (IBD) as the standard vaccination schedule. Blood sample was taken from the brachial vein of birds on 42nd day. A significant decrease in antibody titer against sheep RBC was revealed in the nano-manganese 100 and 150 groups compared to the control group. In addition, the antibody titers against IB and ND were significantly lower in the all nano-manganese groups compared to the control group. No significant difference was observed for the antibody titer against AI and oxidative stress indices among the experimental groups. The findings in the present study suggested that nano-manganese at 50.00, 100 and 150 mg kg⁻¹ levels might suppress humoral immune response in broilers which should be taken into consideration in supplementation.

Introduction

Among the trace minerals commonly used in poultry, manganese is of particular interest in broilers because of its essential roles in metabolism, skeletal growth, enzyme activities and immune responses. Due to its nutritional significance, NRC recommended 60.00 ppm of manganese for broilers. Manganese is important for sustained activity of superoxide dismutase which is vital for antimicrobial function of immune cells. Therefore, higher levels of manganese are supplemented in diets for enhancing immune response in broiler. However, like all essential trace elements, manganese can be toxic when provided at levels in excess of the biological requirement. Fortification of diets with excess manganese could result in generation of free radicals, inactivation of antioxidant enzymes, impairment of immune response and antagonism affecting other trace elements bioavailability. Thus, using high levels of manganese in diet may mask the advantages of supplementation. Indeed, based on the level of supplementation in the diet, manganese could result in beneficial or detrimental effects on immune function, antioxidative defense, mineral uptake and performance of broiler.

With the recent development of nanotechnology, nano-trace elements have attracted widespread attention because nanometer particulates exhibit novel characteristics such as a large surface area, high surface activity, high catalytic efficiency, strong adsorbing ability, and low toxicity. The high activity and efficiency of nano-trace elements could act as a double-edged sword and nano-trace elements can be toxic when the provided levels exceed biological requirements. For example, it has been reported that a high level of nano-selenium supplementation decreased the immune response and antioxidant activity in broilers. Hence, it is important to investigate the possible effects of nano-trace elements at different levels. On a study performed by Lotfi et al. effects of dietary nano- and micro- manganese on growth, performance and bone characteristics of broilers were investigated. Broiler chickens were assigned into different groups, each group were given a diet having a different...
acromolecules such as enzymes and proteins. Therefore, the administration of antioxidants might be beneficial to increase the effectiveness of immune response. Immune cells are vital components of the immune response that kill pathogens by oxygen-dependent mechanisms. Oxygen-dependent mechanisms are initiated by the process of phagocytosis or by perturbation of the cell membrane and are dependent on a membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This enzyme complex is responsible for the production of reactive oxygen species (ROS) after immune stimulation.

ROS play a key role in host defense against pathogens, however, when generated at high levels they can result in oxidative damage. An excessive production of ROS during the immune response can result in oxidative stress if not effectively counteracted by the organism’s antioxidant defenses. ROS can elicit widespread damage to cells and macromolecules such as enzymes and polyunsaturated membrane lipids. In this regard, administration of antioxidants might be beneficial to limit the oxidative damage. Birds exploit a wide range of enzymatic and non-enzymatic substances to cope with oxidants. Micronutrients are antioxidants that have received much attention in birds.

Materials and Methods

Birds, groups and sampling. A total number of 160 1-day-old broiler chicks (Ross 308) were randomly assigned into four groups with three replicates, 13 or 14 birds per replicate. Birds were fed the same basal diet supplemented with nano-manganese oxide, as 0.00 (control group), 50.00, 100, or 150 mg kg⁻¹ of diet. To meet the nutrient requirements of the birds during the experimental period (1-42 days), typical corn-soybean meal-based diets were formulated to meet NRC requirements (Table 1). The size of the nano-manganese (Merck, Darmstadt, Germany) was measured by Particle Size Analyzer (Vasco3; Cordouan Technologies, Pessac, France). The size of the nano-manganese was 30.00 to 150 nm, and the median size was 76.00 nm. The temperature was maintained at 33.00 ± 1.00 °C which was gradually decreased 2.00 °C each week until reaching 20.00 °C and kept at that level. A 24-hr light regimen was conducted throughout the 42-day trial. The birds were vaccinated against avian influenza (AI), Newcastle disease (ND), infectious bronchitis (IB) and infectious bursal disease (IBD), based on the standard vaccination program. Blood sample was collected from the brachial vein of birds on 42th day. Approximately 5.00 mL of blood without anticoagulant was centrifuged at 1,800 g for 15 min. Serum was collected and stored at ~20.00 °C until analysis.

This study was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad (No. 3/45087, date 10/15/2017).

Immune responses. The effect of supplementing nano-manganese on humoral immune responses was studied via measuring the antibody titers against AI, ND and IB using ELISA (Idexx, Westbrook, USA) and hemagglutination inhibition (HI) assays. In addition, sheep red blood cells (sRBC), a non-pathogenic antigen, were used for evaluating the humoral immune response in broiler chickens. The broilers from each dietary group were injected intramuscularly with 0.20 mL of 10.00% sRBC on the 28th day and blood samples were collected at the end of the experiment (day 42).

Table 1. Composition and nutrient levels of basal diets.

| Items                  | Starter | Finisher |
|------------------------|---------|----------|
| Ingredient (%)         |         |          |
| Corn                   | 50.54   | 63.17    |
| Soybean meal           | 42.00   | 31.00    |
| Soybean oil            | 3.00    | 2.00     |
| Dicalcium phosphate    | 1.80    | 1.40     |
| Calcium carbonate      | 1.36    | 1.13     |
| NaCl                   | 0.30    | 0.30     |
| Premix¹                | 1.00    | 1.00     |
| Composition² (%)       |         |          |
| Protein (%)            | 22.50   | 18.50    |
| Calcium (%)            | 0.90    | 0.85     |
| Phosphorus (%)         | 0.45    | 0.42     |
| Sodium (%)             | 0.17    | 0.17     |
| Lysine                 | 1.25    | 1.15     |
| Methionine             | 0.55    | 0.45     |
| Metabolizable energy (kcal kg⁻¹) | 3.100 | 3.050 |

¹ The premix provided the following per kilogram of diet: Cholecalciferol, 5,000 IU; vitamin A, 24,000 IU; vitamin E, 13,000 IU; vitamin K, 2.50 IU; vitamin B1, 1.00 mg; Riboflavin, 75 mg; Pyridoxine, 3.00 mg; vitamin B12, 15.00 μg folic acid, 250 μg nicotinic acid, 17.50 mg; calcium pantothenate, 12.50 mg; Mn, 60.00 mg; Cu, 13 mg; Zn, 40.00 mg; Se, 0.15 mg and Fe, 75.00 mg.
² Components are based on calculated values.
Subsequently, microhemagglutination activity of serum was estimated and the antibody titers (log2) were measured following the standard procedure.19

**Oxidative stress indices.** Malondialdehyde (MDA), as a marker of lipid peroxidation, was measured in serum samples according to Placer et al.20 Dithionitrobenzoic acid method was used for measurement of reduced glutathione (GSH) in the serum samples.21,22 Ferric reducing ability of plasma (FRAP) assay was used for determination of total antioxidant capacity of the serum samples.22

**Statistical analysis.** SPSS for Windows (version 16.0; IBM Corp., Armonk, USA) was used for statistical analysis with a p value of < 0.05 as statistically significant. One-way ANOVA and Bonferroni tests were used for comparison of oxidative stress indices among the different groups. These data were expressed as mean ± standard error (SE). Non-parametric Kruskal Wallis and Mann Whitney U tests were used for comparison of immunological parameters in the trial groups. Quartiles 1 (25.00%), 2 (50.00%, median), and 3 (75.00%) were the descriptive parameters used for these measures.

**Results**

**Oxidative stress.** Although decreased FRAP concentration in all nano-manganese groups and increased MDA concentration in nano-manganese 50.00 and 100 groups were noted when compared to the control group, no significant differences were observed for oxidative stress indices among the trial groups (Table 2).

**Immune response.** A significant decrease in antibody titer against sRBC was revealed in the nano-manganese 100 and 150 groups compared to the control group (p < 0.01). In addition, the antibody titers against IB and ND were significantly lower in the all nano-manganese groups compared to the control group (p < 0.01). No significant difference was observed for the antibody titer against AI among the trial groups (Table 3).

**Table 2. Oxidative stress indices in broilers fed diets containing different levels of nano-manganese (day 42).**

| Parameters                        | Control         | Nano-manganese (mg kg⁻¹ of diet) |
|-----------------------------------|-----------------|----------------------------------|
|                                  |                 | 50.00 | 100 | 150 |
| Malondialdehyde (nmol mL⁻¹)       | 12.08 ± 1.68    | 13.63 ± 2.26 | 14.06 ± 2.08 | 11.01 ± 1.78 |
| Reduced glutathione (mmol L⁻¹)    | 1.17 ± 0.06     | 1.28 ± 0.04 | 1.18 ± 0.08 | 1.08 ± 0.04  |
| Ferric reducing ability of plasma (mmol L⁻¹) | 1.50 ± 0.04 | 1.45 ± 0.04 | 1.38 ± 0.04 | 1.42 ± 0.04  |

No significant differences were observed between the trial groups (p > 0.05).

**Table 3. Immune responses in broilers fed diets containing different levels of nano-manganese (day 42).**

| Items              | Control | Nano-manganese (mg kg⁻¹ of diet) |
|--------------------|---------|----------------------------------|
|                    |         | 50.00 | 100 | 150 |
|                   | Q1 | Q2 | Q3 | Q1 | Q2 | Q3 | Q1 | Q2 | Q3 | Q1 | Q2 | Q3 |
| sRBC titer         | 4.00 | 5.00 | 5.00 | 4.00 | 5.00 | 6.00 | 3.00 | 4.00 | 4.75 | 3.00 | 4.00 | 4.00 |
| ND titer           | 6.00 | 6.00 | 7.00 | 5.00 | 6.00 | 6.00 | 5.00 | 5.00 | 6.00 | 5.00 | 6.00 | 6.00 |
| IB titer           | 723  | 941  | 1237 | 491  | 671  | 1076 | 399  | 579  | 903  | 478  | 705  | 956 |
| AI titer           | 4.00 | 5.00 | 6.00 | 4.00 | 5.00 | 6.00 | 4.00 | 5.00 | 6.00 | 4.00 | 5.00 | 6.00 |

Q1, Q2 and Q3 = quartiles 25.00, 50.00 (median) and 75.00%, respectively.
sRBC= Sheep red blood cells; ND= Newcastle disease; IB= Infectious bronchitis; AI= Avian influenza.
ab Medians within rows lacking a common superscript letter differ significantly at p < 0.05.

**Discussion**

Manganese has been supplemented in broiler diets to improve skeletal growth and enhance immune responses.1 However, the level of supplementation is too critical since manganese in high levels could result in induction of oxidative stress and impairment of immune response.3,23 Although, nano-trace elements have attracted widespread attention in broiler nutrition, their higher activity and efficiency could act as a double-edged sword. Similar to micro-trace elements, nano-trace elements can be toxic when the provided levels exceed the biological requirements.9 Therefore, it is too important to investigate the effects of different levels of nano-trace elements in different biological systems to make sure about their beneficial effects.

Although the effects of dietary nano-manganese (20.00 - 170 mg kg⁻¹) on growth, performance and bone characteristics have been investigated,10 its effect on oxidative stress status and immune response has not been evaluated in broilers. The results of the present study showed significant decreases in the antibody titer against sRBC in the nano-manganese 100 and 150 groups and in the antibody titers against IB and ND in the all nano-manganese groups compared to the control group (p < 0.01). The effects of micro-manganese on the immune response and oxidative stress status have been reported differently. In the study performed by Yang et al. supplementing the diet with 40.00 - 160 mg kg⁻¹ manganese had no significant effects on lymphocyte proliferation in peripheral blood, ND antibodies titers and relative weight of the spleen.8 Gajula et al. reported that a basal diet of corn–soybean meal supplemented with manganese at 60.00, 120 or 240 mg kg⁻¹ had no significant effect on the antibody titers to sRBC, however, manganese at 120 mg kg⁻¹ increased cell-mediated immune response to phytohemagglutinin. It was stated that enhanced cell-mediated immunity might be related to elevated
production of interleukin-2 and increased function of superoxide dismutase, which is vital for the integrity of macrophages and heterophils.\(^1\) Sunder et al. concluded that the supplementation of manganese at 100 mg kg\(^{-1}\) level was essential for skeletal growth and optimum immune response. Manganese supplementation at 100 mg kg\(^{-1}\) level was as efficient as higher levels (up to 800 ppm). Higher levels of manganese (1,600 mg kg\(^{-1}\) and above) had negative effect on antibody titers against sRBC and cutaneous basophil hypersensitivity to phyto-hemagglutinin in broilers.\(^3\) Similarly in a study performed by Liu et al., it was demonstrated that manganese-supplemented diet containing 600, 900, and 1,800 mg kg\(^{-1}\) decreased iron, zinc and calcium contents in immune organs. In addition, IL-1\(\beta\) and IL-2 mRNA levels in immune organs and IL-1\(\beta\) and IL-2 concentrations in blood serum were decreased following manganese supplementation. The authors stated that magnesium at above mentioned levels can disturb the balance of trace elements in immune organs and induce immune suppression in the molecular level.\(^5\) The results of the present study showed that supplementing nano-manganese at 50.00, 100 and 150 mg kg\(^{-1}\) levels exhibited a similar effect to the supplementation of high levels of micro-manganese in terms of suppressed immune function.

Enhanced oxidative stress, as decreased antioxidant capacity and increased lipid peroxidation, was noted in the broilers supplemented with nano-manganese, although it was statistically insignificant (\(p > 0.05\)). The observed findings in the present study suggested that supplementation of nano-manganese at 50.00, 100 and 150 mg kg\(^{-1}\) levels not only did not reveal antioxidant properties, but resulted in mild oxidative stress. Micro-manganese at 20.00 - 100 mg kg\(^{-1}\) levels revealed antioxidative features and diminished superoxide anions (O\(^{2-}\)), and increased antioxidant activity were noted in shrimps fed manganese-supplemented diets.\(^24\) However, higher levels of manganese showed oxidative properties. In a study performed by Liu et al. cocks were fed either a commercial diet or a manganese-supplemented diet containing 600, 900, and 1,800 mg kg\(^{-1}\) manganese chloride. Diets supplemented with manganese increased MDA concentration but decreased antioxidant enzymes activities (superoxide dismutase, glutathione peroxidase) in blood serum and immune organs (spleen, thymus and bursa of Fabricius). DNA single strand break and DNA-protein crosslink revealed time and dosage effect in lymphocytes of immune organs. It was concluded that manganese supplementation at high levels, above 600 mg kg\(^{-1}\), resulted in oxidative damage of immune system by altering antioxidant defense system, lipid peroxidation and apoptosis that might be responsible to some extent in immune suppression.\(^6\) Several studies have reported that manganese-induced cytotoxicity of immune cells is related to oxidative stress.\(^23,25\) The overproduction of ROS and alterations in antioxidant defense system are the possible mechanisms of the oxidative stress induced by manganese supplementation at high levels. The degree of oxidative stress was sensitive to the manganese concentration.\(^6\) The increase of manganese nanoparticles from 10.00 to 100 mg kg\(^{-1}\) resulted in a decrease in the plasma IgM level, however, caused an increase in plasma MDA concentration in young turkeys.\(^26\) Consequently, although it has a vital role in the antioxidant defense system, a high level of manganese, especially in the form of manganese nanoparticles may induce oxidative stress which may exacerbate apoptosis. Similarly, excessive manganese nanoparticles can alter the balance of trace elements in the immune organs and induce immune suppression such as reduction in the IgM level which has a major function in the primary immune response following exposure to a pathogen.\(^26\)

In conclusion, the present study was the first research in which nano-manganese effects on oxidative stress status and immune response was evaluated in broiler chickens. The observed findings suggested that nano-manganese at 50.00, 100 and 150 mg kg\(^{-1}\) levels suppressed humoral immune response in broilers which could be taken into consideration in supplementation. The mechanism of this effect remains to be further studied. In addition, the effects of nano-manganese lower than 50.00 mg kg\(^{-1}\) on immune response and oxidative stress should also be further investigated.

**Acknowledgments**

This study was supported by research fund of Ferdowsi University of Mashhad. The authors wish to thank technicians who kindly helped us for sample collection in this study.

**Conflict of interest**

The authors declare there is no conflict of interests.

**References**

1. Gajula SS, Chelasi VK, Panda AK, et al. Effect of supplemental inorganic Zn and Mn and their interactions on the performance of broiler chicken, mineral bioavailability, and immune response. Biol Trace Elem Res 2011; 139(2): 177-187.
2. National Research Council. Nutrient requirements of poultry. 9th ed. Washington, USA: National Academy Press 1994; 1-176.
3. Sunder GS, Panda AK, Gopinath NCS, et al. Effect of supplemental manganese on mineral uptake by tissues and immune response in broiler chickens. J Poult Sci 2006; 43(4): 371-377.
4. Yang XJ, Sun XX, Li CY, et al. Effects of copper, iron, zinc, and manganese supplementation in a corn and soybean meal diet on the growth performance, meat quality, and immune responses of broiler chickens. J Appl Poult Res 2011; 20(3): 263-271.
5. Liu X, Li Z, Han C, et al. Effects of dietary manganese on Cu, Fe, Zn, Ca, Se, IL-1β, and IL-2 changes of immune organs in cocks. Biol Trace Elem Res 2012; 148(3): 336-344.
6. Liu X, Li Z, Tie F, et al. Effects of manganese-toxicity on immune-related organs of cocks. Chemosphere 2013; 90(7): 2085-2100.
7. Wang H, Zhang J, Yu H. Elemental selenium at nano size possesses lower toxicity without compromising the fundamental effect on selenoenzymes: comparison with selenomethionine in mice. Free Radic Biol Med 2007; 42(10): 1524-1533.
8. Zhang J, Wang X, Xu T. Elemental selenium at nano size (Nano-Se) as a potential chemopreventive agent with reduced risk of selenium toxicity: comparison with Se-methylselenocysteine in mice. Toxicol Sci 2008; 101(1): 22-31.
9. Cai SJ, Wu CX, Gong LM, et al. Effects of nano-selenium on performance, meat quality, immune function, oxidation resistance, and tissue selenium content in broilers. Poult Sci 2012; 91(10): 2532-2539.
10. Lotfi L, Zaghari M, Zeinoddini S, et al. Comparison dietary nano and micro manganese on broilers performance. In proceedings: 5th International conference on nanotechnology: fundamentals and applications. Prague, Czech Republic: 2014.
11. Costantini D, Möller AP. Does immune response cause oxidative stress in birds? A meta-analysis. Comp Biochem Physiol A Mol Integr Physiol 2009; 153(3): 339-344.
12. Weiss DJ, Ramaiah SK, Walcheck B. Neutrophil distribution and function. In: Weiss DJ, Wardrop KJ (Eds). Schalm’s veterinary hematology. 6th ed. Iowa, USA: Wiley-Blackwell 2010; 268-274.
13. Heidarpour M, Mohri M, Borji H, et al. Oxidative stress and trace elements in camel (Camelus dromedarius) with liver cystic echinococcosis. Vet Parasitol 2012; 187(3-4): 459-463.
14. Heidarpour M, Mohri M, Borji H, et al. Oxidant/antioxidant status in cattle with liver cystic echinococcosis. Vet Parasitol 2013; 195(1-2): 131-135.
15. Mozhdeganloo Z, Moghadam Jafari A, Koohi MK, et al. Methylmercury-induced oxidative stress in rainbow trout (Oncorhynchus mykiss) liver: ameliorating effect of vitamin C. Biol Trace Elem Res 2015; 165(1): 103-109.
16. Mozhdeganloo Z, Moghadam Jafari A, Koohi MK, et al. Permethrin-induced oxidative damage in liver of rainbow trout (Oncorhynchus mykiss) and its attenuation by vitamin C. Iran J Vet Res 2016; 17(1): 31-35.
17. Atayi Z, Borji H, Moazen M, et al. Zataria multiflora would attenuate the hepatotoxicity of long-term albendazole treatment in mice with cystic echinococcosis. Parasitol Int 2018; 67(2): 184-187.
18. Evans P, Halliwell B. Micronutrients: oxidant/antioxidant status. Br J Nutr 2001; 85(Suppl 2): S57-S74.
19. Wegmann TG, Smithies O. A simple hemagglutination system requiring small amounts of red cells and antibodies. Transfusion 1966; 6: 67-73.
20. Placer ZA, Cushman LL, Johnson BC. Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. Anal Biochem 1966; 16(2): 359-364.
21. Hu ML, Dillard CJ. Plasma SH and CSH measurement. Methods Enzymol 1994; 223: 385-387.
22. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. Anal Biochem 1996; 239(1):70-76.
23. Vieira MC, Torronteras R, Córdoba F, et al. Acute toxicity of manganese in goldfish Carassius auratus is associated with oxidative stress and organ specific antioxidant responses. Ecotoxicol Environ Saf 2012; 78: 212-217.
24. Wang HW, Cai DB, Zhao CL, et al. Effects of dietary manganese supplementation on antioxidant enzyme activity in the shrimp (Neocaridina heteropoda). Isr J Aquac 2010; 62: 78-84.
25. Chen CJ, Ou YC, Lin SY, et al. Manganese modulates pro-inflammatory gene expression in activated glia. Neurochem Int 2006; 49(1): 62-71.
26. Jankowski J, Ognik K, Stepniowska A, et al. The effect of manganese nanoparticles on apoptosis and on redox and immune status in the tissues of young turkeys. PLoS One 2018; 13(7): e0201487. doi:10.1371/journal.pone.0201487.