Journal of Human Environment and Health Promotion
Print ISSN: 2476-5481 Online ISSN: 2476-549X

The Impact of *In Ovo* Injection of Organic Manganese on Broiler Breeder Hen Egg Hatchability and Progeny Broilers Productivity

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ARTICLE INFO

Article type:
Original article

Article history:
Received: 7 February 2024
Revised: 30 April 2024
Accepted: 15 May 2024

https://doi.org/10.61186/jhehp.10.4.229

Keywords:

In ovo injection
Growth
Egg
Breast-hematology
Intestinal microflora

ABSTRACT

Background: The aim of this study was to investigate the impact of *in ovo* injection of organic manganese (Mn) on the hatching traits and performance of broiler chickens.
Methods: A total of 320 eggs from the Ross 308 strain, with an average weight of 65 g, were used for the experiment. The birds were randomly divided into four groups: four negative control groups (no injection), one positive control group (*in ovo* injection of 0.272 mL of normal saline solution), Manganese I group (*in ovo* injection of 0.013 mg of organic Mn), and Manganese II group (*in ovo* injection of 0.026 mg of organic Mn).
Results: The injected treatments showed a significant decrease in the percentage of hatched chicks compared to the negative control group ($P < 0.0001$). Furthermore, the *in ovo* injection of Mn caused a significant increase in the final weight of the birds and their feed consumption ($P < 0.0001$). The experimental treatments also affected blood parameters, with blood triglyceride, cholesterol, and lipoproteins levels demonstrating a decrease compared to the negative and positive controls ($P < 0.0001$).
Conclusion: The *in ovo* injection of organic Mn showed favorable results in broiler chickens' performance indicators while showing no impact on immune response and microbial population.

1. Introduction

Growth is one of the complex physiological aspects in the animal kingdom, which includes three stages: embryonic, fetus, and neonatal stages. The growth patterns of birds differ somewhat from those observed in mammals (Ravindran & Abdollahi, 2021). Nutrient consumption in birds begins in the early stages of incubation, with the developing embryo being nourished by albumin and albumen (Pandey et al., 2021). Broiler chickens are one of the main components in the poultry industry, which plays a key role in the production of meat and eggs to meet human protein requirements. In addition to effective management practices for breeding breeder hens, the implementation of appropriate nutritional programs and preparing suitable diets are of paramount importance to maximize chicken production and enhance profitability. Most of the commercial diets are based on corn and soy, which cannot provide enough essential minerals needed by birds. Also, due to the presence of anti-nutritional substances such as phytate, part of the minerals in the diet are unavailable (Alfonso-Avila et al., 2022). The amount of Mn in animals is very low, with an average concentration of 0.9 mg/kg live weight in birds (Shokri et al., 2022). Most body tissues contain small amounts of Mn and the highest concentrations are found in bone, liver, kidney, pancreas, pituitary gland, and reproductive organs (Studer et al., 2022). The biochemical role of Mn was unknown until the discovery of the first manganese-containing metalloprotein (pyruvate carboxylase). The specific role of Mn in the production of cartilage mucopolysaccharides was related to the activation of glycosyl transferase. Then, superoxide dismutase (Cu Mn-SOD) was isolated from chicken liver mitochondria and it was shown that it contained 2 mg/kg of Mn. Severe deficiency and inadequate intake of Mn can lead to the

How to cite: Ghane-Khoshkebijari F, Seidavi A, Bouyeh M. The Impact of *In Ovo* Injection of Organic Manganese on Broiler Breeder Hen Egg Hatchability and Progeny Broilers Productivity. *J Hum Environ Health Promot.* 2024; 10(4): 229-37.

damage of immune system and central nervous system (CNS) dysfunction (Studer et al., 2022). Inadequate dietary Mn in mother hens causes a condition called nutritional chondrodystrophy in the fetus. This condition is characterized by various manifestations, including shortened legs and wings, the development of a parrot-shaped beak (attributable to inappropriate lower jaw growth), the roundness of the head (caused by skull protrusion and inclination), abdominal enlargement (due to impaired yolk absorption), and delayed body growth (Adekanmi, 2021). Mn is an essential low-use element for nutrition, and its deficiency can reduce egg production and eggshell thickness, leading to abnormalities in the ultrastructure and morphology of the eggshell (Noetzold et al., 2020). The health risk assessment of trace elements, including Mn, in fruits and vegetables is well-established (Mahmud et al., 2020). For optimal performance of broiler breeding, the recommended level of Mn in the diet for Ross 308 guide is 120 mg/kg (Eivakpour et al., 2021). The absorption rate of Mn from the digestive system of birds is relatively low. Thus, ensuring the bioavailability of Mn is crucial, as an insufficient supply of this element causes additional pressure on the bone structure (Eivakpour et al., 2021). The most rapid stage of skeleton development in chicks occurs during the early post-hatch period and the first two weeks of the rearing phase when the bones are not fully formed. However, the concentration of Mn, along with other minerals, decreases by the 17th day of incubation, resulting in reduced levels in the egg and embryo. Therefore, the possibility of accessing these substances is limited by the embryo at this stage (Sahr et al., 2020). This limitation, coupled with the critical role of Mn in bone growth, can potentially disrupt bone development. Low mineral intake during the first few days of growth may not be sufficient to meet the needs for cartilage ossification. In addition, the low absorption capacity of minerals from the intestine during this period may aggravate this deficiency (Torres & Korver, 2018). Due to the insufficient mineral reserves in contemporary chicken embryos, various strategies have been proposed to overcome these challenges. *In ovo* injection of nutrients has been suggested as a means to alleviate low nutrient intake during the incubation period. Injecting nutrients into the amniotic fluid during incubation is akin to providing an external diet after hatching, enabling digestion and absorption of the injected nutrients in the intestinal tissues (Jha et al., 2019). As the hatching approaches, the growth rate of the embryo decreases due to limited availability of nutrients and energy. Maternal nutrition is the only source of vitamins for eggs, but deficiencies in the maternal diet or the use of low-quality vitamins can result in embryo mortality during incubation (Goel et al., 2020). During the final stages of incubation, high energy levels are required for proper embryo development. In addition, the delayed access to food after hatching, which often occurs within 24-48 h in broilers, shows the necessity of energy and nutrient storage before hatching to maintain body metabolism and regulate body temperature (Nouri et al., 2018). Several strategies have been proposed to improve performance during the early stages of growth, including hatchery feeding (Momeneh & Torki, 2018) and *in ovo* feeding (Ravindran & Abdollahi, 2021). *In ovo* feeding is a novel nutritional method that provides nutrients for embryonic growth before hatching (Ravindran & Abdollahi, 2021). In recent decades, there has been increasing interest in the use of *in ovo* injection for early feeding in birds (Tabatabaei-Vakili et al., 2021). In this process, nutrients are injected into the embryo inside the egg at different embryonic stages. It has been shown that *in ovo* injection of vitamin supplements is useful for the growth of chicks after birth and increases weight. The substances injected into the egg can actively or passively enter the body of the fetus's body through the amniotic fluid and subsequently be absorbed by various organs before the chick hatches (Arain et al., 2022). Nowadays, dietary multi-mineral and multi-vitamin premixes provide most poultry requirements and there is no positive effect of *in ovo* injection of multi-minerals on broiler breeder and its progeny. However, despite the known positive effects of organic Mn feeding in poultry in comparison to other Mn forms, limited data are available on the effects of *in ovo* injection of organic Mn on broiler breeder and its progeny. Contradictory results have been reported with almost similar concentrations. Hence, it is important to conduct more comprehensive research to investigate the impact of Mn as an effective factor in the enzymatic and structural systems, due to the lack of sufficient research in this area.

2. Materials and Methods

2.1 Animals and diets

This experiment was carried out at the hatchery and broiler farm of Navid Morgh Guilan Company in Rasht, Iran in 2022. To conduct the experiment, 320 fertilized eggs from the Ross 308 strain were used. The average weight of the eggs was 65 ± 1 g, and they were obtained from a 55-week-old flock. The eggs were divided into four treatment groups with eight replications. Each replication consisted of 10 eggs. were used in incubation. The experiment consisted of a 21-day incubation period followed by a 42-day rearing period. The temperature and humidity conditions in the setter (Jamesway Multistage, model PT-100, Canada) were 37.6°C and 56%, during which the eggs underwent incubation for 18 days, and then they were transferred to the hatcher (Jamesway Multistage, model PT-100, Canada) with temperature and humidity of 37°C and 58.5%, respectively, for three days.

2.2 In ovo injection and treatments

The eggs were randomly selected for *in ovo* injection. On the 10th day of hatching, Mn was injected into the amniotic sac. First, the injection site was disinfected with betadine, and a hole was made using a round pin. The needle of a HELMA insulin syringe (syringe, China) was inserted into the hole, with the tip protruding 1 mm from the other side of the

cork. The injection was performed at the desired location. Regarding the method of identifying the injection site, it should be mentioned that the fertilized eggs were taken under the light and the needle was inserted in the middle of the chicken's back, where there were the least blood vessels, to the right and parallel to the shell. The treatments based on published paper of our group (Yaripour et al., 2024) were as follows: Treatment 1: The negative control group without any *in ovo* injection. Treatment 2: The positive control group was injected with 0.272 mL of normal saline solution. Treatment 3: Injected with 0.272 mL of a solution containing organic Mn with a concentration of 47.79 µg/ mL. Each egg received an injection of 0.013 mg (0.13 µg) of organic Mn. Treatment 4: Injected with 0.272 mL of a solution containing organic Mn with a concentration of 95.58 µg/mL (Table 1). Each egg received an injection of 0.026 mg (0.26 µg) of organic. The organic Mn used in this research was Manganese-Glycinate (BASF Germany). It had a purity of 21% and was in powder form. the organic Mn was combined with the amino acid glycine in the form of chelate.

2.3 Measurement of traits of hatching chickens

On the 21st day of incubation, the number of chickens hatched from each experimental group was recorded, and the percentage of hatchability was calculated. The weight of the chicks in each replication was also recorded. After that, the chickens were transferred to the rearing farm separately from the experimental treatments. In addition, the unhatched eggs were examined, and the cause time of embryonic death was recorded for statistical analysis.

Table 1. Summary of the treatments employed to examine the effect of *in ovo* administration of Mn

Treatment number	Treatment	Volume Injected (mL)	Manganese content (mg)	Experimental name groups
1	-	0	0	T1
2	Saline vehicle	0.272	0	T2
3	Manganese	0. 272	0.013	T3
4	Manganese	0. 272	0.026	T4

2.4 Raising conditions

After hatching and taking the desired records, the chickens were transferred to the breeding hall. The chicks were divided into four treatments with four replications, and each replication consisted of 10 birds. The chicks were randomly allocated to the pens corresponding to their experimental treatments. The substrate used in the breeding hall was chaff and the pen was made in such a way that in each pen, access to the feeding and drinking nipples was done easily. A vaccination program was implemented, which included double Newcastle-influenza + Bronchitis clone 4.91 by injection into drinking on the first day, clone vaccine spraying on the eighth day, Gumboro vaccine on the fourteenth and twentieth days, and La Sota vaccine as a drink on the sixteenth and twenty-fourth days. During the breeding period, the experimental site had 16 pens, and litter pens were separated by fences with dimensions of 1 × 1 m. Environmental parameters such as temperature, humidity, light, and ventilation were maintained according to the recommended guidelines of the Ross 308 strain., The amount of food consumed for all treatments was considered freely (at the level of appetite). The composition of the feed and its nutritional value can be seen in Table 2. The lighting program followed the recommendations of the Ross 308 strain (Manual, 2012).

Table 2. Diets of experimental groups

Diet components (g/kg)	1-4 days of age	5-11 days of age	12-19 days of age	20-29 days of age	30-38 days of age	39-42 days of age
Ingredients						
Corn	505	517.5	538	551	565	571
Wheat	20	25	30	35	35	37
Corn gluten feed	29	29	34	45	45	54
Iranian soybean meal (5.44%)	328	327	313	300	287	274
Corn gluten (64%)	35	25	17.5	0	0	0
Fish powder	20	10	0	0	0	0
Calcium carbonate	9	8.5	6.6	6.4	5.9	5.4
D-calcium phosphate	13	12.2	11.1	9.3	7.7	6.4
Bentonite	4.6	5.1	5.85	5.9	6.2	6.5
Soy oil	16	21	25	29.5	31	31
Vitamin supplement	1.25	1.1	1	1	1	1
Mineral supplement	3	2.5	2.5	2.5	2.5	2.5
Salt	1.4	1.5	1.8	1.9	2	2.1
Bicarbonate	2.2	2.2	2.2	2.2	2.2	2.2
DL-methionine	3	2.8	2.7	2.5	2.4	2.2
L-lysine chloride	3.4	3	2.8	2.3	1.9	1.7
L-threonine	1.1	0.9	0.9	0.7	0.5	0.3
L-arginine	0.6	0.3	0.2	0	0	0
Choline chloride 60%	0.85	0.8	0.75	0.7	0.6	0.6
Toxinbinder	2	2	2	2	2	2
Multienzyme (containing phytase)	0.1	1	0.1	0.1	0.1	0.1
Monensin sodium 10%	0	1	1	1	1	0
Acidfire	1.5	1.5	1	1	1	0
Total	1000	1000	1000	1000	1000	1000
Nutrients						
Energy (Kcal/kg)	3025	3060	3100	3125	3150	3175
Protein (%)	23.9	22.6	21.2	20.2	19.3	18.8
Calcium (%)	1.01	0.93	0.79	0.7	0.68	0.63
Available phosphor (%)	0.5	0.47	0.43	0.39	0.38	0.35
Sodium (%)	0.16	0.15	0.15	0.15	0.155	0.16
Lysine (%)	1.43	1.34	1.25	1.17	1.11	1.06
Lysine (digestible ileum) (%)	1.29	1.22	1.14	1.06	1.01	0.97

[1]The amount of vitamins and minerals per kg of the final diet: Vitamin A, 9000 IU; vitamin D3, 3000 IU; vitamin E, 18 IU; vitamin K_3, 3 mg; vitamin B_1 (Thiamine), 1.8 mg; vitamin B_2 (Riboflavin), 6 mg; vitamin B_6 (Pyridoxine), 3 mg; vitamin B_{12} (Cobalamin), 0.012 mg; vitamin B_3 (Niacin), 30 mg; vitamin B_9 (Folic acid), 1 mg; vitamin H_3 or B7 (Biotin), 0.24 mg; vitamin B_5 (Pantothenic acid), 10 mg; 500 mg; Choline,100 mg; Mn, 100 mg; Zinc, 80 mg; Iron, 10 mg; Cu, 1 mg; I,0.2 mg.

2.5 Performance

Feed for each experimental unit was placed in labeled bags indicating the pen number and treatment type. The consumed feed was measured periodically, and any feed losses during the measurement period were taken into account to calculate the corrected feed consumption. At the end of each measurement period, the chickens in each experimental unit were weighed as a group, and their number was recorded. The weight gain of each chicken was calculated in grams for each measurement period. The feed conversion ratio (FCR) was calculated according to the weight gain and feed consumption of each experimental unit in each period.

2.6 Carcass characteristics

In order to determine the carcass characteristics and internal organ weights of the broiler chickens, two chickens from each experimental unit were selected at the end of the 42-day rearing period. The selected chickens had weights close to the average weight. After 4-hour starvation, the chickens were slaughtered, and the following measurements were taken: live weight, full weight, carcass weight with a full stomach, carcass weight with an empty stomach, breast weight, thigh weight, wing weight, abdominal fat weight, pancreas weight, heart weight, gizzard weight, spleen weight, duodenum weight, jejunum weight, ileum weight, and cecum weight. Digital scales with an accuracy of 0.001 g (Kern-PFB, Germany) were used for the measurements.

2.7 Blood constitutes and liver enzymes

On the 42nd day of raising broiler chickens, two chickens were randomly selected from each replicate. Blood was taken from the wing veins of these two birds. The two blood samples from each replicate were mixed and one sample was immediately sent to the laboratory to determine the levels of glucose, total cholesterol, and triglyceride, very low-density lipoproteins (VLDL), high-density lipoproteins (HDL), low-density lipoproteins (LDL), the ratio of total cholesterol to HDL, the ratio of LDL to HDL, and liver enzymes. To measure blood parameters, diagnostic kits of Pars Azmoun company (Iran) were used photometrically. Liver enzymes aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase were also measured with diagnostic kits of Pars Azmoun company and photometrically with a spectrophotometer (223337-S2100 UV-UNICO, USA). Serum cholesterol was also measured by an enzymatic-colorimetric method using a kit from Man Company in Iran.

2.8 Immunity

In order to check humoral immunity, broilers were immunized against sheep red blood cells (SRBC). On days 28 and 36, 0.2 cc of SRBC was injected into the wing veins of two birds in each repetition. On the 35th and 42nd days, blood was collected from the injected chickens, and then the antibody titers against SRBC were measured by the hemagglutination inhibition method. To measure the antibody titer, V micro hemagglutination pellets (MAXWELL-NINGBO FUCHUN CO LTD, China) were used. In order to check the titer of Newcastle (NDV) and influenza (AIV) on days 35 and 42 of each experiment, 2 chickens were selected and blood was taken from their wing veins. The hemagglutination inhibition (HI) test was used based on the OIE standard (MAXWELL-NINGBO FUCHUN CO LTD) to check Newcastle and influenza serum titers. For this purpose, 25 µL of PBS was poured into all the wells, then 25 µL of bird serum was added to the first well and its dilution was done until the last well. It was placed on a mechanical shaker for 1 min and then kept at 25 °C for 30 min. In the next step, 25 µL of 1% red blood cells were added to all the wells, and the microplate was again placed on a mechanical shaker for 15 sec. After this stage, the microplates were kept at 25 ºC for 30 min and then the results were recorded. To measure the response of the cellular immune system of four chickens from each repetition, subcutaneous injections of 0.1 mL of PBS and phytohemagglutinin were performed in the left and right wings, respectively, on the 40th day of the experiment. The thickness of the injection site of the wing before and 18 hours after injection was measured with a caliper, and the difference in thickness after injection of the left wing (saline) and thickness after injection of the right wing (PHA) was considered as a measurement criterion with a caliper (Guanglu, China). Also, the weight of the spleen, thymus, and bursa of Fabricius was measured for two samples of each repetition with a digital scale with an accuracy of 0.001 g (Kern-PFB, Germany).

2.9 Mn concentration in the tibia bone

For analysis of bone mineral concentration, one bone ash sample was selected from each treatment. Using the methods specified by the US-EPA, the samples were dissolved and digested, and the Mn concentration in each ash sample was analyzed using a spectrophotometer method (223337-S2100 UV, Unico, USA). To determine the amount of ash, the bones were dried at 105°C for 24 hours. Then the samples were ground and placed in a porcelain crucible in a furnace at 600 ºC.

2.10 Fatty acid profile of breast muscle

Carcass fatty acid profile was determined by extracting 10 g of breast fat from two broilers from each treatment. At first, the fat samples were well mixed with 100 m L of methanol: chloroform solution (2:1) for about 3-4 h. After that, the samples were filtered and mixed with 25 mL saturated sodium chloride solution in the decanter funnel. In the next step, the chloroform phase containing fat was filtered by a filter paper soaked in anhydrous potassium sulfate. The smoothed sample is dried under vacuum by a rotating operator to leave only fat. After this step, 10 mg of extracted fat was mixed well with 2 mL of potassium hydroxide, 2 mL of normal methanol, and 7 mL of n-hexane, then the resulting samples were centrifuged for 10 min. In the next step, the sample remained stationary for 5 min to separate its upper phase. Then, about one µL of the

supernatant phase was injected to evaluate the profile of fatty acids inside the gas chromatography machine (hp6890-US00000397-Agilent-hp-USA), and the number of fatty acids was expressed as a percentage.

2.11 Cecal microbiota

To determine the microbial population of the cecum of chickens, on the 42nd day of each treatment, two birds whose weight was close to the average of the group were selected and slaughtered after weighing. After opening the abdominal cavity, the cecum was separated with sterile scissors and its contents. Its contents were transferred into sterile microtubes. The contents of two birds of each replication were merged. The microbial population of dominant bacteria including lactic acid bacteria, *Escherichia coli*, and coliform population were studied. To count lactic acid bacteria from de Man, Rogosa, and Sharpe agar culture medium, to count *Escherichia coli* bacteria from EMB-agar (Eosine methylene blue agar) culture medium, and to count coliform bacteria from MacConkey agar was used. Finally, the colonies related to each culture medium were counted as colony-forming units (CFU) in 1 g of sample and the CFU data were reported in Log10 format to be used for data analysis.

2.12 Statistical analysis

The data obtained from the experiment were analyzed in the form of a completely random design. A one-way ANOVA test was applied using SPSS software, and the mean comparisons of the treatments were evaluated using Duncan's multiple-range test.

$Yij = \mu + \alpha i + \varepsilon ij$, Yij = number of observations in the experiment, μ = mean, αi = effect of each treatment, εij = effect of experimental error

3. Results and Discussion

In ovo injection of organic Mn caused a significant decrease in the hatching index of experimental and positive control groups compared to the negative control group (Table 3). However, there was no significant effect of *in ovo* injection on the weight of day-old chicks. Late infection related to the injected treatments was significantly higher (Table 3). The weight gains of birds and the amount of feed consumed in groups 3 and 4 were significantly higher than the negative and positive control groups (Table 3). However, there was no significant difference between the groups injected with Mn. The conversion coefficient did not show any significant difference among the experimental groups. On the other hand, the production index in the groups injected with Mn was significantly higher than the control groups (negative and positive). This resulted in significant differences in final weight and feed consumption in groups 3 and 4. In addition, in terms of production index, groups 3 and 4 were significantly improved compared to both control groups (positive and negative), as shown in Table 3.

Table 3. Effect of *in ovo* injection of different levels of Mn on the mean (± standard error of means) of some hatching traits and subsequent growth, feed intake, and feed conversion ratio

Items	T_1	T_2	T_3	T_4	SEM	*P-value*
Hatchability (%)	95.0 [a]	55.0 [b]	63.750 [b]	66.250 [b]	5.815	0.000
Chick weight (g)	46.485 [a]	44.493 [a]	44.569 [a]	45.297 [a]	0.632	0.118
Chicken for sale	9.125 [a]	5.000 [b]	5.750 [b]	5.875 [b]	0.548	< 0.0001
Cull chick	0.375 [b]	0.500 [b]	0.625 [a]	0.750 [a]	0.261	0.766
Unhatched egg	0.375 [b]	4.500 [a]	3.500 [a]	3.375 [a]	0.582	0.000
Early contamination	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000	1.000
Late contamination	0.000 [b]	1.000 [a]	0.375 [b]	0.375 [b]	0.186	0.007
Broken egg	0.000 [a]	0.000 [a]	0.000 [a]	0.000 [a]	0.000	1.000
inconclusive egg	0.000 [a]	0.500 [a]	0.000 [a]	0.000 [a]	0.000	1.000
Feed Intake (g)						
1-21d	1259.025 [a]	1212.775 [a]	1226.250 [a]	1148.950 [a]	33.972	0.193
22-42d	2670.100 [b]	2911.900 [b]	3587.550 [a]	3404.950 [a]	112.629	0.000
1-42d	3929.125 [b]	4124.500 [b]	4813.600 [a]	4553.900 [a]	99.208	0.000
Weight Gain (g)						
1-21d	935.743 [a]	942.515 [a]	962.063 [a]	907.868 [a]	29.997	0.652
22-42d	1257.043 [b]	1428.200 [b]	1766.500 [a]	1683.875 [a]	60.300	0.000
1-42d	2192.770 [c]	2370.715 [b]	2729.563 [a]	2591.618 [a]	51.841	< 0.0001
Feed Conversion Ratio (g/g)						
1-21d	1.280 [a]	1.280 [a]	1.217 [b]	1.203 [b]	0.008	< 0.0001
22-42d	2.128 [a]	2.038 [ab]	2.033 [b]	2.023 [b]	0.021	0.013
1-42d	1.753 [a]	1.708 [a]	1.747 [a]	1.748 [a]	0.011	0.035
Production Index	304.300 [c]	328.540 [b]	378.250 [a]	359.450 [a]	7.259	< 0.0001
Feed price per kg of live weight (Rials/kg)	290395.940 [a]	284171.425 [a]	280069.055 [a]	284393.590[a]	3138.053	0.194

* T1: Negative control (without injection), T2: Positive control (injection of 0.272 mL of normal saline solution), T3: Mn (0.013 mg), T4: Mn (0.026 mg). [a, b] Means within each row of treatments with no common superscript differ significantly at $P < 0.05$.

In ovo injection of organic Mn did not have a significant effect on carcass characteristics and relative weight of body organs such as the bursa of Fabricius, spleen, liver, heart, and different parts of the digestive system including the cecum, duodenum, pancreas, jejunum, and ileum ($P = 0.5$) (Tables 4 and 5). *In ovo* injection of organic Mn improved certain blood parameters. The levels of cholesterol metabolites, triglycerides, VLDL, LDL, and HDL lipoproteins decreased

significantly under the influence of injected organic Mn (Table 5). Blood glucose levels also increased significantly in the Mn 3 group (0.013 mg) compared to all experimental groups ($P < 0.0001$).

Table 4. Effect of *in ovo* injection of different levels of Mnon the mean (± standard error of means) of carcass characteristics of broiler chickens at market weight

Items	T_1	T_2	T_3	T_4	SEM	*P-value*
Live body weight (g)	2238.125 [b]	2400.625 [b]	2866.875 [a]	2693.125 [b]	54.401	< 0.0001
Defeather body weight (g)	2093.875 [b]	2313.750 [b]	2771.250 [a]	2589.375 [a]	61.877	< 0.0001
Full abdomen carcass weight (g)	1793.125 [b]	1993.750 [b]	2422.500 [a]	2250.000 [b]	54.261	< 0.0001
Empty abdomen carcass weight (g)	1491.875 [a]	1640.625 [a]	2003.750 [a]	1868.750 [a]	48.395	< 0.0001
Breast weight (g)	558.650 [b]	589.155 [b]	731.325 [a]	671.375 [a]	21.945	0.000
Relative weight of breast (%)	31.043 [a]	25.740 [a]	30.230 [a]	29.875 [a]	1.783	0.206
Drumsticks (thighs) weight (g)	429.907 [b]	484.582 [b]	577.975 [a]	508.270 [a]	38.865	0.110
Relative weight of drumsticks (thighs) (%)	24.000 [a]	25.565 [a]	23.875 [a]	19.485 [a]	3.097	0.563
Wings weight (g)	120.300 [b]	131.610 [b]	364.770 [a]	151.575 [a]	100.454	0.310
Relative weight of wings (%)	6.710 [a]	6.595 [a]	6.953 [a]	6.745 [a]	0.096	0.119
Abdominal fat weight (g)	36.390 [b]	35.378 [b]	46.803 [a]	39.818 [a]	3.306	0.115
Relative weight of abdominal fat (%)	2.025 [a]	1.765 [a]	1.940 [a]	1.755 [a]	0.147	0.508
Pancreas weight (g)	4.763 [a]	5.435 [a]	5.175 [a]	4.547 [a]	0.430	0.486
Relative weight of pancreas (%)	0.263 [a]	0.270 [a]	0.213 [a]	0.205 [a]	0.023	0.158
Gizzard weight (g)	22.390 [a]	23.243 [a]	21.798 [a]	26.713 [a]	1.720	0.233
Relative weight of gizzard (%)	1.255 [a]	1.180 [ab]	0.903 [a]	1.078 [a]	0.097	0.112
Heart weight (g)	10.765 [a]	10.300 [a]	11.455 [a]	10.555 [a]	0.821	0.780
Relative weight of heart (%)	0.600 [a]	0.520 [a]	0.470 [a]	0.472 [a]	0.035	0.073
Proventriculus weight (g)	8.330 [a]	9.385 [a]	10.495 [a]	9.655 [a]	0.579	0.121
Relative weight of proventriculus (%)	0.460 [a]	0.465 [a]	0.435 [a]	0.433 [a]	0.027	0.766
Crop weight (g)	8.082 [a]	8.650 [a]	8.962 [a]	9.090 [a]	0.966	0.883
Relative weight of crop (%)	0.445 [a]	0.430 [a]	0.375 [a]	0.405 [a]	0.042	0.661

* T1: Negative control (without injection), T2: Positive control (injection of 0.272 mL of normal saline solution), T3: Mn (0.013 mg), T4: Mn (0.026 mg). [a, b] Means within each row of treatments with no common superscript differ significantly at $P < 0.05$.

Table 5. Effect of *in ovo* injection of different levels of Mn on the mean (± standard error of means) of gastro-intestinal characteristics and blood parameters at market weight

Items	T_1	T_2	T_3	T_4	SEM	*P-value*
Gastro-intestinal characteristics						
Duodenum weight (g)	15.550 [a]	14.980 [a]	15.657 [a]	15.618 [a]	1.345	0.982
Relative weight of duodenum (%)	0.875 [a]	0.750 [ab]	0.645 [a]	0.695 [a]	0.072	0.184
Jejunum weight (g)	34.063 [a]	41.800 [a]	39.168 [a]	36.973 [a]	2.623	0.248
Relative weight of jejunum (%)	1.903 [ab]	2.073 [a]	1.620 [a]	1.643 [a]	0.123	0.067
Ileum weight (g)	26.770 [a]	30.705 [a]	35.718 [a]	30.863 [a]	2.294	0.105
Relative weight of ileum (%)	1.483 [a]	1.533 [a]	1.463 [a]	1.382 [a]	0.116	0.833
cecum weight (g)	13.588 [a]	10.045 [a]	13.438 [a]	12.377 [a]	1.270	0.229
Relative weight of cecum (%)	0.768 [a]	0.505 [b]	0.548 [a]	0.550 [a]	0.067	0.066
Blood parameters						
Glucose (mg/dl)	54.025 [b]	62.575 [b]	132.250 [a]	62.575 [b]	4.390	<0.0001
Total cholesterol (mg/dl)	152.225 [a]	139.975 [b]	110.325 [b]	124.575 [c]	2.526	<0.0001
Triglycerides (mg/dl)	140.900 [a]	134.075 [a]	103.100 [b]	107.075 [b]	5.592	0.001
VLDL (mg/dl)	28.325 [a]	27.025 [a]	20.650 [b]	21.475 [b]	1.141	0.001
HDL (mg/dl)	42.400 [a]	40.200 [ab]	34.875 [c]	36.600 [c]	0.693	<0.0001
LDL (mg/dl)	72.125 [a]	64.825 [b]	49.850 [c]	58.925 [b]	1.954	<0.0001
LDL/HDL	1.698 [a]	1.610 [a]	1.425 [b]	1.608 [a]	0.040	0.003
SGOT (AST) (U/L)	237.250 [b]	227.500 [b]	235.250 [b]	275.000 [a]	5.843	0.000
SGPT (ALT) (U/L)	41.950 [a]	35.425 [b]	57.500 [a]	57.500 [a]	2.216	<0.0001
Cholesterol/HDL	3.585 [a]	3.483 [a]	3.170 [c]	3.403 [b]	0.049	0.000
Alkaline phosphate (U/L)	7180.000[a]	8025.000 [a]	5147.500 [c]	6330.000 [bc]	445.706	0.004

* T1: Negative control (without injection), T2: Positive control (injection of 0.272 mL of normal saline solution), T3: Mn (0.013 mg), T4: Mn (0.026 mg). [a, b] Means within each row of treatments with no common superscript differ significantly at $P < 0.05$. VLDL: very-low-density lipoprotein, HDL: high-density lipoprotein, LDL: low-density lipoprotein, SGOT: serum glutamic-oxaloacetic transaminase, AST: aspartate aminotransferase, SGPT: serum glutamic pyruvic transaminase, ALT: alanine aminotransferase.

Mn 4 treatment (0.026 mg) significantly improved the performance of the aspartate transaminase enzyme compared to other experimental groups ($P < 0.0001$) (Table 5). Also, in the treatment of three Mn (0.013 mg), the level of alanine transaminase increased significantly compared to the experimental groups ($P < 0.0001$). In addition, treatment 3 Mn (0.013 mg) had lower levels of alkaline phosphate than the control group (negative and positive) ($P = 0.004$). Also, the results related to the injection of phytohemagglutinin in the right and left leg of broiler chickens and the degree of swelling caused by it in the skin membrane of the leg show that the experimental treatments did not have a significant difference between the positive and negative control groups ($P = 0.15$) (Table 6). Statistically significant differences were

not observed in the immune response index in terms of the titer of antibodies produced against Newcastle disease and influenza viruses, as well as the intestinal microbial population between the experimental treatments and negative and positive control groups, except for the significantly higher levels of coliform in the third treatment (Table 6). Heterophils, eosinophils, and monocytes are phagocytes, which digest the invading microbes. Increased free radicals oxidize and destroy biological cells, causing many disorders in the intestinal tissues (Ocak et al., 2008). The presence of dietary antioxidants can solve problems associated with intestinal disease and improve microbial functional properties (Nosrati et al., 2017). The results of the fatty acid profile of the breast muscle are shown in Table 7. The numbers obtained from the studies show that there is no significant relationship between the injected Mn and the fatty acid profile of the breast muscle. In all the investigated fatty acids, the numbers between the positive and negative control groups and the groups injected with Mn are very close to each other. Similar studies have not been reported in this regard. Regarding the concentration of Mn in the tibia of the left leg, the results of the investigation between the experimental treatments and the negative and positive control groups are shown in Table 7. The resulting numbers show that there is no significant effect of *in ovo* injection on Mn concentration in the tibia, which can be attributed to the injected Mn concentration and the day of injection. In the present study, the *in ovo* injection of Mn caused a significant decrease in the hatching index, which is consistent with the results of Geng *et al.* (2022). One of the possible causes of the observed drop due to the significance of late contamination of unhatched eggs can be improper injection and human error caused by injection, which causes contamination inside the developing embryo. On the other hand, the results obtained from the performance indicators of birds regarding weight gain, final weight, and feed consumption show that the performance is improved under the influence of organic Mn. This shows that corn and soy-based diets in breeder hens cannot meet the requirements and seem insufficient to store this micronutrient in the egg for the growth and development of the embryo and supplementation in this way can bring beneficial effects to this industry. (Geng et al., 2022) did not observe any change in functional indices with *in ovo* injection of different levels of Mn (6.25, 12.5, 25, and 50 µg). As we know, bone is the richest source of Mn in the body, which contains 3-4 µg/g of Mn, followed by the liver with 2 µg/g. The absorption of Mn from the digestive system is weak, and one of the reasons is the formation of natural chelates, which reduces its availability. Mn is excreted through feces, although its rate of excretion is influenced by dietary Mn concentration. The ability to access inorganic Mn through the diet varies and depends on the compounds containing it (mineral salts or artificial chelates) and is between 40% and 80%. In addition, the presence of antagonistic relationships, such as the effect of phosphorus on Mn absorption is another factor that limits the availability of Mn in the diet. On the other hand, Mn plays a role in metabolic processes, including oxidative phosphorylation in mitochondria, fatty acid synthesis, and cholesterol metabolism. *In ovo* injection of Mn and manganese-methionine nanoparticles separately and in the amount of 1 mL per egg reported that *in ovo* injection of different sources of Mn improved the growth of broiler chickens and the highest weight gain in the entire rearing period was related to the treatment of Mn nanoparticles. These results are consistent with those of our study. In addition, the best conversion coefficient was observed for treatments injected with nano-manganese and manganese-methionine. Also, the length of the tibia and the fresh and dry weight of the tibia at the age of 21 and 42 days were significantly higher in the treatment injected with Mn nanoparticles. On the other hand, none of the experimental treatments had a significant effect on carcass components at the age of 42 days although, in other studies, the highest percentage of carcass and cookable breast muscle weight was related to the treatment containing Mn nanoparticles (Talab et al., 2019). One of the possible causes in this regard could be the composition of the ration and the concentration of Mn in the experimental rations, which caused such differences in the results; things such as the levels of phosphorus, calcium, and iron in the ration can affect the availability of Mn. In addition, the injection dose in the research was much lower and could be the main cause of such contradictory results. Mn feeding in broilers revealed that the use of these sources in corn and soy-based diets had a significant effect on body weight, feed consumption, and FCR. This effect is due to the supply of Mn required for growth by dietary ingredients. Therefore, the use of higher amounts of Mn in broiler diets can provide the required Mn for enhanced growth and bone development (Groff-Urayama et al., 2023). However, it is important to consider the potential side effects and risks associated with Mn supplementation, as noted by Underwood and Suttle (Underwood & Suttle, 1999). Studies involving chicks indicate that liver, kidney, and skeletal Mn levels are responsive to dietary Mn, particularly when the Mn range is magnified tenfold (Halpin & Baker, 1986). Excessive Mn intake leading to increased tissue Mn levels is likely an indication of disrupted homeostasis rather than successful storage. Because of the large contribution of the skeleton to body mass, even a small increase in bone Mn concentration could constitute a significant passive reserve (O'Dell & Sunde, 1997). There is evidence of interference with iron absorption and a reduction in hemoglobin synthesis at high Mn intakes. The adverse interaction probably arises from competition with iron for binding sites on transferrin and other carriers, and the precise toxic threshold is therefore likely to depend on dietary iron concentration. In general, the present study shows that the *in ovo* injection of Mn in broiler eggs improves performance indicators such as final weight and feed consumption. Further research on higher concentrations *in ovo* Mn concentrations will provide insights into the density of this element in the bone, along with the measurement of mentioned indicators. In addition, investigating the preventive effects of Mn on various types of lameness in meat herds, including pros and common forms, would be valuable.

Table 6. Effect of *in ovo* injection of different levels of Mn on the mean (± standard error of means) of immune parameters and cecal microflora (CFU/g) at market weight

Items	T_1	T_2	T_3	T_4	SEM	*P-value*
Immune parameters						
Titer against influenza at 21 days (lg2)	4.500 [a]	4.250 [a]	4.000 [a]	3.750 [a]	0.368	0.533
Titer against influenza at 28 days (lg2)	3.000 [a]	3.000 [a]	2.250 [a]	3.500 [a]	0.339	0.233
Titer against the first injection of Newcastle after 7 days (lg2)	4.500 [a]	4.500 [a]	6.250 [a]	5.500 [ab]	0.451	0.047
Titer against second injection of Newcastle after 7 days (lg2)	3.750 [a]	3.750 [a]	4.000 [a]	3.750 [a]	0.217	0.152
The number of antibodies produced against sheep red blood cells (TSRBS)	4.50 [ab]	3.500 [b]	3.750 [a]	3.500 [a]	0.280	0.081
Inflation index of the skin of the foot membrane						
Right foot before phytohemagglutinin injection (mL)	18.18 [a]	17.515 [a]	18.153 [a]	17.043 [a]	1.004	0.825
Right foot after phytohemagglutinin injection (mL)	19.79 [a]	19.723 [a]	21.078 [a]	19.980 [a]	0.675	0.480
left foot before phytohemagglutinin injection (mL)	18.25 [a]	17.110 [a]	18.060 [a]	17.448 [a]	0.921	0.802
left foot after phytohemagglutinin injection (mL)	20.72 [a]	20.878 [a]	22.058 [a]	20.995 [a]	0.832	0.670
Thymus weight (g)	3.510 [a]	4.573 [a]	4.868 [a]	4.343 [a]	0.666	0.534
Relative weight of thymus (%)	0.195 [a]	0.230 [a]	0.205 [a]	0.195 [a]	0.031	0.831
Liver weight (g)	54.26 [a]	53.062 [a]	58.675 [a]	56.225 [a]	3.178	0.629
Relative weight of liver (%)	2.570 [a]	2.355 [a]	2.425 [a]	2.490 [a]	0.243	0.933
Spleen weight (g)	3.000 [a]	3.008 [a]	3.415 [a]	3.020 [a]	0.326	0.763
Relative weight of spleen (%)	0.170 [a]	0.150 [a]	0.163 [a]	0.237 [a]	0.054	0.673
Bursa of Fabricius weight (g)	1.375 [a]	2.460 [a]	1.880 [a]	2.140 [a]	0.352	0.220
Relative weight of bursa of Fabricius (%)	0.075 [a]	0.118 [a]	0.078 [a]	0.155 [a]	0.035	0.365
Cecal microflora						
Coliform	4.500 [b]	4.500 [b]	6.250 [a]	5.500 [ab]	0.451	0.047
Escherichia coli	4.500 [a]	4.250 [a]	4.000 [a]	3.750 [a]	0.368	0.533
Lactic acid bacteria	3.750 [a]	3.750 [a]	4.000 [a]	3.250 [a]	0.217	0.152

* T1: Negative control (without injection), T2: Positive control (injection of 0.272 mL of normal saline solution), T3: Mn (0.013 mg), T4: Mn (0.026 mg). [a, b] Means within each row of treatments with no common superscript differ significantly at $P < 0.05$.

Table 7. Effect of *in ovo* injection of different levels of Mn on the mean of the meat fatty acids profile and Mn consternation in the tibia of broiler chickens (%)

Items	T_1	T_2	T_3	T_4
Meat fatty acids profile				
Myristic acid (C14:0)	0.385	0.45	0.425	0.405
Pentadecanoic acid (C15:0)	0.055	0.085	0.065	0.065
Palmitic acid (C16:0)	24.71	24.44	23.33	22.94
Heptadecanoic acid (C17:0)	0.055	0.065	0.055	0.04
Stearic acid (C18:0)	6.75	6.70	5.78	5.51
Arachidic acid (C20:0)	0.18	0.19	0.15	0.175
Heneicosanoic acid (C21:0)	0.135	0.22	0.155	0.15
Behenic acid or Docosanoic acid (C22:0)	0.185	0.235	0.215	0.205
Myristoleic acid (C14:1)	0.055	0.12	0.1	0.085
Palmitoleic acid (C16:1)	3.88	3.80	4.29	4.27
Heptadecenoic acid (C17:1)	0	0	0.07	0.055
Oleic acid (C18:1, n-9)	38.04	36.43	36.90	36.53
Elaidic acid (C18:1t)	0	0.07	0.055	0.025
Gondoic acid or Eicosenoic acids (C20:1)	0.095	0.175	0.065	0.1
Linoleic acid (C18:2, n-6)	23.04	24.04	25.38	26.07
Linolenic acid (C18:3)	1.59	1.87	2.05	2.17
Dihomo-gamma linoleic acid (C20:3)	0.615	0.915	0.645	0.695
Mn consternation in the tibia of broiler				
Concentration (mg/kg)	4.64	5.22	4.93	5.44

* T1: Negative control (without injection), T2: Positive control (injection of 0.272 mL of normal saline solution), T3: Mn (0.013 mg), T4: Mn (0.026 mg).

4. Conclusion

In ovo injection of organic Mn showed favorable results in the performance of broiler chickens, specifically in terms of feed consumption, weight gain, and final body weight. However, no effect on immune response and microbial population was observed. Conversely, the causes of the negative effect of hatching chickens should be removed to bring an acceptable economic return to the producer. This study has certain limitations including a small number of replicates and the absence of related gene expression. Moreover, the strengths of the study are the investigation of comprehensive parameters related to productivity and lifecycle of broilers in comparison to other existing studies.

Authors' Contributions

Farhad Ghane-Khoshkebijari: Data curator; Formal analysis; Investigation; Writing-original draft. Alireza Seidavi: Conceptualization; Project administration; Supervision; Validation; Writing-review and editing. Mehrdad Bouyeh: Conceptualization; Project administration; Validation; Writing-review and editing.

Funding

This work was funded by Rasht Branch, Islamic Azad University. (grant number 6298.1).

Conflicts of Interest

There is no conflict of interest.

Acknowledgements

This article does not contain any acknowledgment.

Ethical considerations

The use and care of birds in this study were approved by the Rasht Branch, Islamic Azad University, Rasht, Iran. All experimental procedures were also approved by the Rasht Branch, Islamic Azad University, Rasht, Iran (11748259376765014001623001 8). Care was taken to minimize the number of birds used.

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