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Research Article

Effects of Organic and Inorganic Manganese Supplementation on Bone Characteristics, Immune Response to Vaccine and Oxidative Stress Status in Broiler Reared Under High Stocking Density [1] [2]

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Abstract

This study was carried out to determine the effects of organic and inorganic manganese (Mn) at different levels supplemented to diets of the broilers under stocking density stress on bone characteristics, immune response to vaccine and oxidative stress. A total of 1000, day-old broiler chicks were divided into one control group and nine treatment groups, each consisting of 100 chicks. They were randomly located at a density of 0.064 kg/m^2 until reaching seven days of age and in the compartments with 2 m^2 space at a density of 60 kg/m^2 until they are 49 days old. The control group was fed the basal diet only, whereas experimental groups were fed with basal diet supplemented with organic Mn (6.25, 12.5 and 25 mg/kg of Mn-methionine), inorganic Mn (12.5, 25 and 50 mg/kg Mn-oxide) and organic + inorganic Mn (3.125+6.25, 6.25+12.5 and 12.5+25 mg/kg Mn-methionine + Mn-oxide). There were no changes on days 25 and 49 in terms of physical properties such as humerus, femur and tibia as well as tibia fracture resistance and tibial metaphyseal chondral tissue lenght in all experimental groups (P>0.05). Tibia bone elasticity was higher in the 50 mg/kg Mn-oxide group compared to the control and other groups on day 49 (P<0.05). The serum calcium level on day 25 increased in the 6.25 and 12.5 mg/kg Mn-methionine and 6.25+12.5 mg/kg Mn-methionine + Mn-oxide groups (P<0.01). Antibody titre against Newcastle Disease Virus in Mn-methionine group on day 25 and titer against Infectious Bursal Disease in Mn-methionine and Mn-oxide groups on day 49 were higher compared to the control group (P<0.01). Serum levels of malondialdehyde and nitric oxide on day 49 decreased in all exprimental groups, whereas serum glutathione level increased in the same groups (P<0.01). As a result, dietary Mn supplementation to broilers hosted at 60 kg/m^2 of stocking density increased immune response and antioxidant activity by reducing the impact of density stress; yet, did not affect the physical properties of the bone. The effects of organic and inorganic forms of Mn on the examined properties were concluded to be similar.

Keywords: *Stocking density stress, Organic Mn, Inorganic Mn, Bone characteristic, Immune response, Oxidative stres, Broiler*

Sıklığa Maruz Bırakılmış Broylerlerde Rasyona Organik ve Inorganik Manganez İlavesinin Kemik Özellikleri, Aşılara Karşı İmmun Yanıt ve Oksidatif Stres Durumuna Etkileri

Özet

Araştırma sıklık stresi altındaki broylerlerde rasyona farklı düzeylerde organik ve inorganik manganez (Mn) ilave edilmesinin kemik özellikleri, aşılara karşı immun yanıt ve oksidatif stres üzerine etkilerinin incelenmesi amacıyla yapıldı. Araştırmada toplam 1000 adet günlük yaşta broyler civciv her biri 100 civcivden oluşan 1 kontrol ve 9 deneme grubuna ayrıldı. Civcivler 7 günlük yaşa kadar 0.064 kg/m^2'lik ve 49 günlük yaşa kadar 2 m^2'lik bölmelerde 60 kg/m^2 sıklıkta barındırıldı. Kontrol grubu temel rasyonla, deneme grupları ise temel rasyona sırasıyla 6.25, 12.5 ve 25 mg/kg düzeylerinde organik Mn (Mn-metiyonin), 12.5, 25 ve 50 mg/kg inorganik Mn (Mn-oksit), 3.125+6.25, 6.25+12.5 ve 12.5+25 mg/kg organic + inorganik Mn (Mn-metiyonin + Mn-oksit) ilave edilerek beslendi. Araştırmanın 25. ve 49. günlerinde broylerlerde humerus, femur ve tibiaya ait incelenen fiziksel özellikler ile tibia kırılma mukavemetinin ve tibial metafizyal kıkırdak doku uzunluğutüm deneme gruplarında değişmediği belirlendi (P>0.05). Tibia kemiği elastikiyeti 49. günde 50 mg/kg Mn-oksit grubunda kontrol ve diğer gruplara göre yüksek bulundu (P<0.05). Serum kalsiyum düzeyinin 25. günde 6.25 ve 12.5 mg/kg Mn-metiyonin ile 6.25+12.5 mg/kg Mn-metiyonin + Mn-oksit gruplarında arttığı belirlendi (P<0.01). Newcastle hastalığı virusuna karşı antikor titresinin 25. günde Mn-metiyonin, Enfeksiyöz Bursal hastalık virusuna karşı antikor titresinin ise 49. günde Mn-metiyonin ve Mn-oksit gruplarında kontrol grubuna göre yüksek olduğu belirlendi (P<0.01). Serumda 49. gündeki malondialdehit ve nitrik oksit düzeyleri tüm deneme gruplarında düşük iken, glutasyon düzeyinin aynı gruplarda arttığı belirlendi (P<0.01). Sonuç olarak 60 kg/m^2 yerleşim sıklığında barındırılan broylerlerde rasyona Mn ilavesinin sıklık stresinin etkisini azaltarak immun yanıtı ve antioksidan aktiviteyi artırdığı, fiziksel kemik özelliklerini etkilemediği belirlendi. Organik ve inorganik Mn formlarının incelenen özellikler üzerine etkisinin benzer olduğu kanaatine varıldı.

Anahtar sözcükler: *Sıklık stresi, Organik Mn, İnorganik Mn, Kemik özelliği, İmmun yanıt, Oksidatif stress, Broyler*

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INTRODUCTION

In the second half of the twentieth century, global poultry meat production has developed more rapidly than any farm animal meat production [1]. The poultry meat production which occurred as 103 million tons in 2010 is predicted to reach 122.5 million tons in 2020 [2]. This growth springs from modern broiler genotypes obtained from intense selections applied for juvenile growth rate, breast-meat yield and feed efficiency [3-5], and intensive production systems where high-tech is used [6].

Acoording to European Commission Directive (2007/43/EC) optimum stocking densityis 33-39 kg/m^2 when provided that the owner or keeper complies with the requirements setout this legislation. In addition, this density can be increased by a maximum of 3 kg/m^2 when further requirement are provided in the same legislation. However, when the stocking density increases the movement of broiler chickens are restricted largely in the overcrowded conditions and the opportunity to exercise required for bone development is reduced [2]. Due to the genetic rehabilitation, bones of broilers develop more slowly than the muscle tissue, and the lack of exercise resulting from high stocking density affects locomotor system reversely. Thus, major foot and leg problems and welfare loss owing to the increase in stocking density were reported [7-9]. Especially, the impacts of high stocking density in broilers on the foot health, lameness or gait disturbance, tibial dischondroplasia, gait scores, carcass bruising and scratching were reported in many researches. At finaly, the leg abnormalities result in culling, mortality and economic losses [6,10,11].

It is quite clear that, in the perspective of cost-benefit analysis, stocking density is of vital importance in producing more broiler chickens per unit area [7,11-13]. In this context, the findings that the environmental conditions are more effective on the health and welfare of the broiler chickens than the stocking density are of vital importance for the economic future of white meat industry [10,14-16]. Therefore, reducing the reverse impacts of high stocking density by the modification of environmental conditions, and increasing the upper limit set by the relevant regulations for the stocking density form a significant option [17].

In the researches conducted on the feed compositions, the importance of dietary Mn for the commercial broiler sector was especially expressed [17-21], One of the problems caused by the stocking density is the increasing lipid peroxidation in the chickens [22,23]. It specifically involves in MnSOD structure of the Manganese. In the case of oxidative stress, MnSOD steps in to protect cell mitochondrias from the degrading effects of free oxygen radicals [24]. The opportunities of benefiting from minerals taking part in enzymatic activities (Orjinase, piruvate, carboxilase and Mn superoxide dismutase etc.) are being examined in order to reduce these problems [10]. The effects of dietary nickel [25], calcium, copper, zinc and iron [26,27] in broiler diets have been examined. The importance of manganese (Mn) has been gradually increasing, since it is significant for bone formation, enzyme function and amino acid metabolism in the poultry [27,28].

In recent years, many supplementary organic Mn sources have been produced and used in feeding animals; yet, economic reasons overweights in determining dietary Mn forms being supplemented in conventional broiler diets. The notifications about the sources of Mn additives or the doses administered are conflicting. According to Berta et al.[29] and El-Husseiny et al.[30] organic forms of Mn are superior to inorganic forms of Mn in terms of utility, whereas there is no difference between the forms of Mn according to Bertechini and Hossain [30] and Conly et al.[20]. The importance of manganese (Mn) has been gradually increasing since it is significant for bone formation, enzyme function and amino acid metabolism in the poultry [28,31].

Therefore, the objective of this study was to determine the effects of organic and inorganic Mn supplementation to the diet of broilers grown under stocking density stress on bone characteristics, immune response and oxidative stress status.

MATERIAL and METHODS

Birds and Management: This study was conducted from May to July at the Afyon Kocatepe University Animal Research and Application Center with the approval of the University Ethics Committee (AKUHADYEK-37-2008). A total of 1000, day-old broiler chicks were used in this study. They were divided into one control group and nine treatment groups, each consisting of 100 chicks. Each group was subdivided into four sub-groups (replicates) with 25 chicks in each replicate; all subgroups were randomly placed in cages with 1 m^2 space. Birds were raised until 49 days of age under the same housing conditions and at a stocking density of 60 kg/m^2. Thus, a total 40 cages were used having 25 birds in each in conformity according to the management guide for Hubbard broilers [21]. Feed and water were provided *ad libitum*. A vaccination program for broilers was designed as day 0 with inactive Infectious Bursal Disease (IBD) + Newcastle Disease (ND) vaccine (Gumbopest, Merial RTA, subcutaneously), day 7 with live ND vaccine (Nobilis ND Lasota, Intervet, in drinking water) and infectious bronchitis vaccine (Nobilis, Intervet, in drinking water), day 14 with live IBD vaccine (Bursine Plus, Ford Dodge-Refarm, in drinking water), and day 21 with live ND vaccine (Nobilis ND Lasota, Intervet, in drinking water) vaccines.

Dietary Treatments: The control group was fed a basal diet including pre-dominantly of corn, soybean meal and full fat soybean *(Table 1)*. The experimental groups were fed basal diets containing three different diet treatments

(organic Mn, inorganic Mn and organic plus inorganic combination) with three different dose levels for each treatment. Each dietary treatment was comprised of 100 birds which were further sub-divided into four replicates, each replicate containing 25 birds. The organic Mn groups were fed diets supplemented with 6.25, 12.5 and 25 mg/kg Mn-methionine, inorganic Mn groups were offered diets containing 12.5, 25 and 50 mg/kg Mn-oxide and organic + inorganic Mn groups were fed diets containing 3.125 mg/kg Mn-methionine + 6.25 mg/kg Mn-oxide, 6.25 mg/kg Mn-methionine + 12.5 mg/kg Mn-oxide and 12.5 mg/kg Mn-methionine + 25 mg/kg Mn-oxide. The nutrient composition of the basal diet *(Table 1)*, including crude protein and calcium was determined according to the AOAC [32]. The metabolisable energy (ME) of the basal diet was estimated using the Carpenter and Clegg equation [33]. The available phosphorus, lysine, methionine and threonine levels of the basal diet were calculated according to the diet guide for Hubbard broilers [34].

Table 1. *Composition of the basal diets used in different periods (%)*
Tablo 1. *Farklı dönemlerde kullanılan temel rasyonun bileşimi (%)*

Ingredients	Starter (0 to12 day)	Grower (12 to 28 day)	Finisher (28 to 49 day)
Corn	47.37	50.44	51.85
Soybean meal	24.65	17.70	14.82
Full fat soybean	15.00	18.00	17.00
Poultry meal	4.00	4.00	5.00
Meat bone meal	2.50	4.00	4.00
Vegetable oil	3.50	3.52	4.91
Limestone	0.73	0.59	0.61
Dicalcium phosphate	0.86	0.46	0.35
DL-Methionine	0.36	0.33	0.34
Sodium bicarbonate	0.30	0.25	0.31
Vitamin premix*	0.20	0.20	0.20
Mineral premix**	0.10	0.10	0.15
Salt	0.14	0.12	0.07
L-Lysine	0.13	0.13	0.23
Enzyme***	0.10	0.10	0.10
Anticoccidial	0.06	0.06	0.06
Chemical composition			
ME**** (kcal/kg)	3050	3200	3360
Crude protein, %	23.20	22.70	21.80
Calcium, %	1.00	1.00	1.00
Phosphorus (available), %	0.49	0.49	0.49
Lysine, %	1.50	1.40	1.40
Methionine, %	0.75	0.70	0.70
Threonin, %	0.997	0.884	0.902

** Vitamin premix (Rovimix 124-F) provides per 2.5 kg of diets: 15.000.000 IU vitamin A, 1.500.000 IU vitamin D, 50.000 vitamin E, 5.000 mg vitamin K_3, 3.000 mg vitamin B_1, 6.000 mg vitamin B_2, 25.000 mg niacin, 12.000 mg Ca-D Pantothenate, 5.000 mg vitamin B_6, 30 mg vitamin B_{12}, 1.000 mg folic acid, 125 mg D-biotin, 300.000 mg choline chloride, 300.000 L-lysine; ** Mineral premix provides per kg of diets: 80.000 mg manganese, 30.000 mg ferric, 60.000 mg zinc, 5.000 mg cupper, 500 mg cobalt, 2.000 mg iodine, 235.680 mg calcium carbonate; *** Enzyme (Optimise M): 5.000 BU/g endo-1.3 beta-glucanai 5.000 BXU/g endo-1.4-ksilinase, 500 FYT/g phytase; ****Estimated according to the Carpenter and Clegg equation*

Bone Characteristics: At the age of 25 and 49 days, 8 birds were slaughtered (4 male and 4 female) from each group. The fresh weights of the left tibia, femur and humerus bones (after muscle, cartilage and membranes were removed) of the chicken were measured with 0.01 g precise digital scales; vertical diameters were measured using digital compass; their volumes were determined. Also, fracturing resistance of left tibia bones was determined via three-point break test performed in accordance to Park et al.[35]. Bone density was determined via the values collected with the help of a measuring cylinder and bone weight values. The Tibia weight/length index was calculated by dividing the tibia weight by its length [36].

In order to evaluate the tibial metaphyseal chondral tissue lenght, right tibial bones of 8 slaughtered birds were separated for histopathological examinations. By opening articulation genus in rear extremities, muscle and other tissues were removed. Cutting tibias from the center, extremitas proximalises were taken into buffered formalin solution. After being detected fixed for 72 h, the tissues were taken into Decastro (buffered 3% nitric acid) solution and decalcification was projected. Solutions were renewed every three days. Once the tissues softened, they were washed in running water for 48 h and embedded in paraffin after routine tissue monitoring. They were cut with microtome and stained with hematoxylin eosin, were examined in the light microscope. Slides were examined via DP20 camera set up on Olympus B51 microscope. Via Cell A program, from epiphyseal plates of the bones towards distal (metaphysis), the cartilage tissue was measured upto the area where it transforms into bone tissue *(Fig. 1)*. The length of the cartilage tissue were scored as <1000 μ=1; 1001-1500 μ=2; 1501-2000 μ=3; >2001 μ=4. The ratio of leg abnormalities in the groups were determined using percentage of severely lame birds that clinically lameness or unable to walk.

Serum Parameters: On days 25 and 49 of this study, serums obtained from the blood samples of 8 birds (4 male and 4 female) from each group were stored at -18ºC for determination of serum malondialdehyde (MDA), glutathione (GSH), nitric oxide (NO), calcium (Ca) and phosphorus (P) levels and antibody titers against Infectious Bursal Disease (IBD) and Newcastle Disease (ND). The levels of serum MDA, GSH and NO were determined by the methods described by Drappier et al.[37], Beutler et al.[38] and Miranda et al.[39], respectively. Total specific antibody production in serum against IBD and ND was performed by using commercial ELISA kits and in accordance to Puthpongsiriporn and Scheideler [40]. Serum Ca and P levels were determined with 300 Alobat Alcycon-auto analyzer.

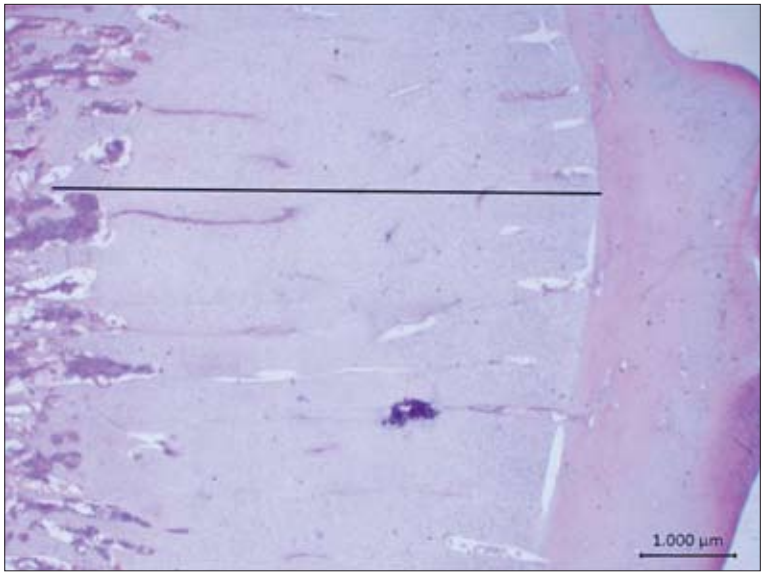

Fig 1. The line in the picture shows that the cartilage tissue was measured for metaphyseal chondral tissue in tibia, HE

Şekil 1. Resimdeki çizgi tibiadaki metafizial kıkırdak doku için ölçülen doku uzunluğunu göstermektedir, HE

Statistical Analysis: Differences among group values with regard to all parameters were examined by analysis of variance for all values and the significance of differences between groups tested by Duncan's test. Besides, Mn-methionine, Mn oxide, mixture groups and the control group each of which composing of three groups were considered as single dimensions, and the Contrast test was applied in order for each dimension to be contrasted to the block of other dimensions. A value of P<0.05 was considered as the limit for statistical significance.

RESULTS

Bone Characteristics: On days 25 and 49 of the research, physical properties such as weight and length of humerus, weight and length of femur; weight, length, thickness, density and volume of tibia and tibiotarsi index *(Table 2)* as well as tibia fracture resistance *(Table 3)* and tibial metaphyseal chondral tissue length *(Table 4)* did not change (P>0.05) between the control and experimental groups. Tibia bone elasticity was found higher in the group supplemented with 50 mg/kg Mn-oxide compared to the control and other Mn supplemented groups on day 49 (P<0.05; *Table 3*).

Serum Parameters: In this study, It was determined that antibody titre against ND in Mn-methionine group on day 25 and titer against IBD in Mn-methionine and Mn-oxide groups on day 49 were higher compared to the control group (P<0.01). At the same time, ND titer on day 25 and IBD titer on day 49 increased in the 12.5 mg/kg Mn-oxide group and in the 12.5+25 mg/kg Mn-methionine + Mn-oxide groups, respectively (P<0.01). Serum Ca level increased in the 6.25 and 12.5 mg/kg Mn-methionine and 6.25+12.5 mg/kg Mn-methionine + Mn-oxide groups compared to the control group on day 25 (P<0.01), whereas Ca level on day 49 and P level on day 25 and 49 in serum did not change in all experimental groups (P>0.05; *Table 5*).

According to the Contrast test results; In terms of ND antibody level, there were significant differences between Mn-methionine group and the other groups (P<0.01; 25 and 49 day-old chicks); Mn-methionine + Mn-oxide group and the other groups (P<0.001; 25 day-old chicks); the control group and the other groups (P<0.001; 25 and 49 day-old chicks). In terms of IBD antibody level, there were significant differences between Mn-methionine group and the other groups (P<0.05; 49 day-old chicks); the control group and the other groups (P<0.01; 49 day-old chicks). In terms of Serum Ca level, there were significant differences between Mn-methionine group and the other groups (P<0.01; 25 day-old chicks); Mn-oxide group and the other groups (p<0.05; 25day-old chicks), the control group and the other groups (P<0.01; 25 day-old chicks).

Serum GSH and NO levels on days 25 and 49 as well as serum MDA level on day 49 showed significant variations between the experimental groups. Serum levels of MDA and NO on day 49 decreased in the group supplemented with Mn, whereas serum GSH level increased in the same groups (P<0.01). Serum NO level on day 25 was lower (P<0.05) in the 25 mg/kg Mn-oxide and Mn-methionine + Mn-oxide groups compared to the control group *(Table 6)*.

According to the Contrast test results; In terms of MDA level there were significant differences between Mn-methionine + Mn-oxide group and the other groups (P<0.001; 49 day-old chickens), control group and the other groups (P<0.01; 49 day-old chickens). In terms of GSH level, the differences were significant between Mn-oxide group and the other groups (P<0.01; 25 day-old chickens), Mn-methionine + Mn-oxide group and the other groups (P<0.01; 49 day-old chickens), the control group and the other groups (P<0.001; 25 and 49 day-old chickens). In terms of NO level, the differences were significant between Mn-oxide group and the other groups (P<0.05; 25 day-old chickens), Mn-methionine + Mn-oxide

Table 2. *Effects of organic and inorganic Mn supplementation to diets of the broilers under stocking density stress on some bone characteristics*
Tablo 2. *Sıklık stresine maruz bırakılmış broyler rasyonlarına organik ve inorganik Mn ilavesinin bazı kemik özelliklerine etkisi*

Parameters	Days	Control	Mn-methionine (mg/kg)			Mn-oxide (mg/kg)			Mn-methionine + Mn-oxide (mg/kg)			SE	P
			6.25	12.5	25.0	12.5	25	50	3.125+6.25	6.25+12.5	12.5+25.0		
Humerus weight (g)	25	3.41	3.30	2.73	3.05	3.04	3.27	3.40	3.11	4.05	3.24	0.09	0.24
	49	6.55	7.84	7.93	8.58	7.42	7.89	7.77	7.60	7.42	6.36	0.21	0.36
Humerus length (mm)	25	50.35	52.37	48.84	49.19	49.97	51.11	51.68	49.01	51.91	49.26	0.36	0.23
	49	67.97	69.55	70.62	71.21	69.83	69.33	70.85	68.97	68.75	67.58	0.51	0.82
Femur weight (g)	25	5.66	5.31	5.26	5.98	5.64	5.15	5.73	6.13	5.58	5.31	0.09	0.38
	49	10.06	10.57	12.42	11.28	11.59	10.20	11.56	9.98	12.13	9.97	0.32	0.57
Femur length (mm)	25	47.77	54.71	54.48	55.75	55.07	54.51	55.87	55.54	54.70	54.51	0.67	0.26
	49	71.33	73.26	75.09	73.38	72.72	72.29	73.90	71.82	74.03	73.46	0.47	0.85
Tibia weight (g)	25	8.03	7.72	7.28	8.57	7.71	7.79	8.76	8.72	8.25	7.98	0.13	0.18
	49	14.20	14.26	14.95	16.50	16.35	14.38	17.19	12.57	15.65	13.33	0.48	0.56
Tibia length (mm)	25	75.83	75.67	74.99	77.99	76.53	75.44	77.78	77.81	76.49	76.01	0.31	0.34
	49	101.35	101.60	103.36	102.64	104.28	117.04	103.81	97.23	102.97	111.46	1.87	0.62
Tibia thickness (mm)	25	6.01	6.50	6.56	6.12	5.92	6.11	5.74	5.97	5.82	5.92	0.06	0.08
	49	7.88	8.24	8.25	8.36	8.27	7.90	8.33	8.70	8.35	8.02	0.09	0.72
Tibia density	49	1.58	1.99	1.67	2.08	2.21	1.73	1.80	1.92	2.09	1.75	0.08	0.80
Tibia volume (cm^3)	49	7.61	7.42	8.60	7.79	6.92	7.08	8.48	6.81	7.03	7.76	0.30	0.94
TibioWt/lenght index, mg/mm	25	105.92	101.82	97.05	109,50	100.75	102.98	112.48	111.79	107,83	104,56	1.41	0.26
	49	137.79	138.81	143.11	158.39	155.92	127.00	164.50	128.78	150.71	124.34	3.94	0.30

Table 3. *Effects of organic and inorganic Mn supplementation to diets of the broilers under stocking density stress on fracture characteristics of tibia bone*
Tablo 3. *Sıklık stresine maruz bırakılmış broyler rasyonlarına organik ve inorganik Mn ilavesinin tibia kemiği kırılma özellikleri üzerine etkisi*

Parameters	Days	Control	Mn-methionine (mg/kg)			Mn-oxide (mg/kg)			Mn-methionine + Mn-oxide (mg/kg)			SE	P
			6.25	12.5	25.0	12.5	25	50	3.125+6.25	6.25+12.5	12.5+25.0		
Maximum fracture height (N)	25	208.98	218.46	265.64	259.70	190.80	223.95	215.20	226.05	208.51	222.41	5.68	0.13
	49	231.79	288.00	283.96	332.44	283.69	269.00	285.82	356.66	270.85	250.76	11.18	0.39
Tibia bone elasticity (mm/s)	25	1.69	1.72	1.67	1.68	1.57	1.70	1.73	1.51	1.76	1.96	0.03	0.23
	49	1.97^{bc}	2.06^{bc}	2.07^{bc}	2.40^{ab}	1.89^{c}	1.98^{bc}	2.64^{a}	2.01^{bc}	2.14^{c}	1.88^{c}	0.05	0.04
Tibia fracture resistance (N/mm) MPa	25	73.62	67.35	72.91	87.00	71.37	76.61	82.96	79.88	80.74	83.91	1.72	0.15
	49	60.36	65.81	64.39	67.50	63.93	68.75	61.07	67.85	58.55	63.05	1.64	0.96
Tibia fracture resistance Kgf (N/9.81)	25	21.30	22.27	27.08	26.47	19.44	22.83	21.94	23.04	21.26	22.67	0.58	0.13
	49	23.62	29.36	28.95	33.89	28.92	27.42	29.13	36.36	27.61	25.57	1.14	0.40

Letters (a, b, c) in the same line indicate significant differences between different letters; Tibia bone flexibility (mm/second): The collapse amount of the bone during the fracturing process; millimeter/second; Tibia bone resistance (kgf (N/9.81): Maximum force that the bone could endure during the fracturing; kgf, N/mm

group and the other groups (P<0.05; 49 day-old chickens), the control group and the other groups (P<0.05; 25 and 49 day-old chickens).

DISCUSSION

In this study, physical properties of the bones such as humerus, femur and tibia and Ca levels in serum (other than Ca level on day 25), as well as tibia fracturing properties (other than tibia elasticity on day 49) were not affected from Mn supplementation. It was observed that the values of tibia fracturing resistance were found to have numerically increased in Mn-methionine groups. Also, tibia fracturing resistance was detected to be the lowest in the control group which Mn supplementation was not applied. The fact that a statistically significant difference was not observed in the present study may be due to the high standard error resulting from small sampling size (n). As a matter of fact our finding corresponds with the research results asserting that Mn contributes to bone properties. Ruff and Hughes [41] and El Husseiny et al.[30] also reported similar results and the researchers indicated that dietary

Table 4. *Effects of organic and inorganic Mn supplementation to diets of the broilers under stocking density stress on tibial metaphyseal chondral tissue lenght, distribution of tibial metaphyseal chondral tissue length score and ratio of leg abnormalities*

Tablo 4. *Sıklık stresine maruz bırakılmış broyler rasyonlarına organik ve inorganik Mn ilavesinin tibialarda metefizial kıkırdak doku uzunlugu, metafizial kıkırdak doku skorlarının dağılımı ve bacak problemleri oranı üzerine etkisi*

Groups		25th day					49th day					
		Cartilage Tissue Length	Cartilage Tissue Length Score				Cartilage Tissue Length	Cartilage Tissue Length Severity Score				Leg Abnormalities
		Mean (µm)	1	2	3	4	Mean (µm)	1	2	3	4	%
Control		2154.22		1	1	2	3020.85		3	2	3	5.60
Mn- meth	6.25	2382.90		1		6	1676.16	1	2	3	2	2.59
	12.5	2109.89		2		4	1934.09	1	6		1	2.73
	25	3204.90			1	6	1261.89	1	4	3		1.26
Mn-oxide	12.5	2032.29	2			5	1536.79		2	5		9.70
	25	2020.12			2	2	1613.41	1	4	1	2	4.17
	50	2499.51		1		5	1567.28		5	2	2	2.63
Mn-meth +Mn-oxide	3.125+6.25	2320.49		1	2	4	1687.73		4	2	2	2.50
	6.25+12.5	2422.62			2	4	1646.31	1	1	4	2	0
	12.5+25.0	2541.83		1		4	1411.41	1	4	3		2.70
SEM		140.73		0.11			143.94			0.01		
P		0.767		0.919			0.328			0.538		

Severity score of tibial metaphyseal chondral tissue: <1000µ=1; 1001-1500µ=2; 1501-2000µ=3; >2001µ=4

Table 5. *Effects of organic and inorganic Mn supplementation to diets of the broilers under stocking density stress on ND and IBD antibody titers and Ca (mg/dl) ve P (mg/dl) levels in serum*

Tablo 5. *Sıklık stresine maruz bırakılmış broyler rasyonlarına organik ve inorganik Mn ilavesinin serum ND ve IBD antikor titresi ile Ca (mg/dl) ve P (mg/dl) düzeyi üzerine etkisi*

Parameters	Days	Control	Mn-methionine (mg/kg)			Mn-oxide (mg/kg)			Mn-methionine+Mn-oxide (mg/kg)			SE	P
			6.25	12.5	25.0	12.5	25	50	3.125+6.25	6.25+12.5	12.5+25.0		
ND	25	2308^{c}	7287^{b}	11352^{a}	7390^{b}	7829^{b}	5259^{bc}	5313^{bc}	2320^{c}	2164^{c}	2638^{c}	457.2	0.00
	49	9266^{b}	11177^{ab}	15591^{a}	14873^{a}	11221^{ab}	14095^{ab}	9080^{b}	10391^{ab}	12260^{ab}	11672^{ab}	540.5	0.06
IBD	25	15956	16675	16207	13973	13868	14168	17738	13737	12304	11646	668.4	0.55
	49	10492^{c}	15195^{a}	17417^{a}	15903^{a}	14758^{a}	14663^{ab}	11175^{ab}	11782^{c}	16041^{cb}	15511^{a}	397.9	0.00
Ca	25	4.89^{d}	6.11^{b}	7.19^{a}	5.22^{cd}	4.90^{d}	5.07^{cd}	5.37^{bcd}	4.76^{d}	5.91^{bc}	5.16^{cd}	0.11	0.00
	49	5.91	7.73	5.96	7.54	7.50	7.76	6.65	5.98	7.00	6.16	0.22	0.28
P	25	4.24	4.07	4.19	4.18	3.80	3.92	3.72	3.57	3.70	4.22	0.07	0.33
	49	5.98	7.85	5.99	6.84	6.29	7.05	5.82	5.51	6.16	5.89	0.21	0.34

Letters (a, b, c, d) in the same line indicate significant differences between different letters; Data are means of 8 replicate cages consisting of 12 birds per replicate cage

Table 6. *Effects of organic and inorganic Mn supplementation to diets of the broilers under stocking density stress on oxidative stress status*

Tablo 6. *Sıklık stresine maruz bırakılmış broyler rasyonlarına organik ve inorganik Mn ilavesinin oksidatif stres durumu üzerine etkisi*

Parameters	Days	Control	Mn-methionine (mg/kg)			Mn-oxide (mg/kg)			Mn-methionine+Mn-oxide (mg/kg)			SE	P
			6.25	12.5	25.0	12.5	25	50	3.125+6.25	6.25+12.5	12.5+25.0		
MDA	25	280.62	252.25	258.50	250.00	247.25	260.37	240.25	212.62	227.75	225.25	6.09	0.40
	49	3.71^{a}	2.89^{bcd}	3.06^{bcd}	2.62^{d}	3.28^{abc}	2.91^{bcd}	2.77^{cd}	3.03^{bcd}	2.88^{bcd}	3.35^{a}	0.05	0.01
GSH	25	22.47^{c}	24.76^{b}	25.87^{ab}	24.87^{b}	25.75^{ab}	26.77^{a}	25.06^{ab}	25.14^{ab}	26.01^{ab}	26.04^{ab}	0.20	0.00
	49	17.43^{d}	20.32^{c}	23.24^{ab}	25.22^{a}	22.33^{c}	25.09^{ab}	23.67^{ab}	23.74^{ab}	24.93^{ab}	26.07^{a}	0.38	0.00
NO	25	13.62^{a}	13.06^{ab}	11.88^{abc}	11.73^{abc}	12.91^{ab}	11.03^{bc}	11.56^{abc}	11.35^{bc}	11.44^{bc}	10.63^{c}	0.22	0.04
	49	12.80^{a}	11.28^{b}	11.39^{b}	10.06^{bc}	9.85^{c}	10.10^{bc}	10.40^{bc}	11.20^{b}	10.22^{bc}	9.39^{c}	0.18	0.00

Letters (a, b, c, d) in the same line indicate significant differences between different letters

manganese improved bone features. Also, we found the largest tibial metaphyseal chondral tissue in control group at 49 days of age *(Table 4)*. In general, it has been known that increase in the lenght of tibial metaphyseal chondral tissue is related to a predisposing factor for tibial dyschondroplasia [42,43]. It can be argued that regarding to those results, Manganese suplementation into diet decreased the incidence of tibial dyschondroplasia in broilers reared under high stockind density.

The positive effect of manganese on tibia in this study has suggested that it enhanced Ca accumulation in tibia. In this study, Ca levels in the groups Mn was supplemented were lower than the control group without Mn, and that difference reflected on the Contrast test. Sunder et al.[44] also reported that 100 ppm of dietary Mn supplementation contributed to foot-leg health. However, these differences in calcium did not reflect upon P level. Therefore, it must be considered that dietary Mn supplementation especially during the growth period might distrupt the ratio of Ca and P. Furthermore, while the number of the chickens with clinical lameness or foot discomfort was at acceptable levels (2-3%) in the groups other than the group with 12.5 mg/kg of inorganic Mn and the control group despite the bone parameters and increasing serum Ca level, this rate was found to be numerically higher (5.6%) in the control group.

The present study revealed that broilers supplemented with dietary Mn responded to ND and IBD vaccines by producing antibodies at higher levels than chickens fed on the control feed with no Mn supplementation. Both ND and IBD antibody levels elevated more particularly in Mn methionine groups when compared to other groups *(Table 5)*. These findings suggest that Mn enhances the immune system in broilers. Especially organic manganese has a more powerful impact on that. Similarly, Sunder et al.[44] reported that dietary Mn supplementation at the level of 100 mg/kg enhanced immunity. Gajula et al.[45] also asserted that immune response became swifter as dietary Mn level increased. Moreover, as the stress caused by the exposure to stocking density was reduced to some extent by the feed with Mn supplementation, the immunity of the chickens in the control group with no Mn supplementation may have been affected from this stress more. Similarly, Bozkurt et al.[46] observed that the *bursa of fabricius* weight did not change in the groups supplemented with organic and inorganic Mn.

In this study, serum MDA and NO levels of the broilers fed with dietary Mn supplementation were found to be lower compared to the control group *(Table 6)*. Similarly, Bulbul et al.[47] reported that organic or inorganic dietary Mn supplementation reduced serum MDA. However, Lu et al.[48] reported that dietary Mn supplementation did not affect serum MDA level in broilers. In this study, high MDA level in the control group may be related to abdominal fat amount. The deterioration in oxidant-antioxidant balance in favor of antioxidants in the control group may have led to high MDA and NO levels causing high feed consumption and fat accumulation, consequently. In fact, it was observed that less abdominal fat was numerically or statistically detected in broilers fed with Mn supplemented feed than in the control group except for the group feeding with 25 mg/kg Mn-oxide [46]. It has also been reported that redundant free fatty acid intake excessing antioxidant capacity may increase oxidative stress by leading to lipid peroxidation and also bring moleculer features of fat tissue into the forefront as a major cause for oxidative stress [49]. Lu et al.[48] also reported that 100 mg/kg of dietary Mn supplementation lowered abdominal fat amount. Furthermore, Mn supplementation to the diet above the rate determined for broilers by NRC may suggest that Mn has inhibited the activities of enzymes involving in lipid peroxidation.

As a result, it was observed that dietary Mn supplementation enhanced immune response and antioxidant activity by reducing the effect of density stress for broilers having been hosted at 60 kg/m^2 stocking density; slightly increased ossification in tibia while it did not affect the physical properties of the bone or bone resistance. Considering the levels used at the study, organic and inorganic forms of Mn have similar impacts on examined properties; yet, further research on the impacts of higher doses of Mn on ossification is required.

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