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Iron Loaded Chitooligosaccharide Nanoparticles Reduces Incidence of Bacterial Chondronecrosis with Osteomyelitis in Broiler Chickens

Research Article

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ABSTRACT

The current study was conducted to investigate the effects of dietary iron-loaded chitooligosacharide nanoparticles (Fe-CNP) on occurrence of bacterial chondronecrosis with osteomyelitis (BCO) in broiler chickens. Four hundred eighty day-old chicks (Ross 308) were assigned to four treatments with six replicates of 20 birds. All chicks were reared on wire flooring system up to 42 days of age. The control diet was formulated to meet the Aviagen recommendations except for iron supplement. The experimental groups were contain: 10 mg/kg Fe from Fe-CNP (group Fe-CNP), 20 mg/kg Fe from $FeSO_4$ (group $FeSO_4$) and 20 mg/kg Fe from $FeSO_4$ + CNP (group $FeSO_4$+CNP). Results showed a significant increase in production efficiency factor, weight gain and FCR in all Fe supplemented groups ($P<0.05$) compared to the control. Feed intake was not differed significantly between groups ($P>0.05$). Serum tumor necrosis factor-alpha (TNF-α) level decreased significantly by Fe-CNP, whereas serum immunoglobulin G (IgG) level increased significantly in this group ($P<0.05$). The prevalence of impaired gait scores (GS≥3) in live birds in day 42 did not show a significant difference between dietary treatments ($P>0.05$). Lame broilers evaluation showed that lower control and Fe-CNP had lower impaired gaits ($P<0.05$). Supplementation of $FeSO_4$ significantly increased the percentage of femur and tibia lesions ($P<0.05$). Leg evaluation of live broilers showed that Fe-CNP and control groups exhibited a higher incidence of normal femur (NF) and a lower percentage of transitional femur necrosis (FHT) and total femur lesions (All F; $P<0.05$). Femoral head necrosis (FHN) and FHS were not affected significantly by dietary treatments ($P>0.05$). Totally, Fe-CNP supplement, by improving immune system, reduced the prevalence of BCO lesions in broiler chickens.

KEY WORDS bacterial chondronecrosis, broiler chicken, chitooligisaccharide, iron, lameness.

INTRODUCTION

During two past decades, genetic selection for rapid growth has increased the susceptibility of broilers to lameness due to bacterial chondronecrosis with osteomyelitis (BCO). Recent studies have demonstrated that totally BCO affects 1.5% of broiler chickens grown to yield market in the US (Dinev, 2009). The BCO refers to necrotic degeneration and infection of proximal ends of femur and tibia which can accompany with spondylitis (Dinev, 2009). The pathogenesis of BCO is complex, briefly mechanical forces due to high growth rate, causes osteochondrotic microfractures in the immature cartilaginous growth plates of femur and tibia. After that, the vascular tissue of growth plates have truncated by osteochondrotic clefts and results in local ischemia and necrosis. Pathogenic bacteria enter the circulation via translocation through the intestine epithelium, and the fenestrated endothelium of occluded vessels facilitate the passage of hematogenously distributed bacteria into the hypertrophic zone of the growth plate. Colonization of bacteria in osteochondrotic clefts results in infective and necrotic foci (McNamee and Smyth, 2000). Osteochondrosis is not re-

sponsible for clinical signs of lameness per se, but the bacterial infection of necrotic zone of bones results in BCO and lameness (Smeltzer and Gillaspy, 2000; Wideman and Prisby, 2013). Intestine and growth plates are the sources of bacterial translocation and colonization respectively. So, maintaining of health and performance of intestine is critical in BCO prevention programs. Dietary iron in some cases by destruction of intestinal epithelium and disturbance of the eubiosis of microflora can participate in BCO occurrence. Iron is an essential nutrient which participate in oxygen and electron transfer and is involved in structure of hemoglobin, myoglobin, cytochromes and also it modulate immune system (Arredondo and Núñez, 2005). On the other hand, some evidences revealed that, excessive iron can induces variable degrees of toxicity both in cellular (Houglum *et al*. 1998; Bacon *et al*. 1983; Toyokuni, 2002) and gastrointestinal (Toblli *et al*. 2008) levels. The role of iron in occurrence of BCO in broiler chickens may contribute to oxidative properties of iron in intestine lumen, which in turn facilitates bacterial translocation. Dramatically dietary iron by catalyzing free radicals production can induces intestinal inflammation (Tompkins *et al*. 2001; Nielsen *et al*. 2007; Balamurugan *et al*. 2010; Zimmermann *et al*. 2010; Werner *et al*. 2011) and gastrointestinal toxicity (Toblli *et al*. 2008). Free oxygen radicals and activated neutrophils by disrupting the cytoskeleton of the mucosa, can increase mucosal permeability (Wiest and Rath, 2003), therefore leading to bacterial translocation. Practically iron is supplemented in the form of sulfate, carbonate and oxide in poultry diets (Bao *et al*. 2007). According to the Aviagen (2014), iron requirement of broiler chicken is 40 mg/kg that 20 mg of it should supply as mineral supplement. The use of iron chelators to prevention deleterious effects of mineral iron in gastrointestinal tract have been demonstrated in some studies. In intestinal inflammation, iron chelators agent have employed in order to reducing luminal free iron (Millar *et al*. 2000) and thereby it prevents deleterious effects of iron specially on mucosal tissue. Toblli *et al*. (2008) evaluated acute and late toxicity of three different oral iron supplements (ferrous sulfate, iron amino chelate and iron-polymaltose complex) in gastrointestinal tract and liver in rats. Results showed that ferrous sulfate revealed acute toxicity, and early gastrointestinal tract and liver toxicity. In current study iron was bounded with chitosan derivatives to increase immunity, prevention of oxidative stress induction by iron in intestine lumen and prophylactic prevention of bacterial translocation and occurrence of BCO in broiler chickens.

MATERIALS AND METHODS

Chitooligosaccharide, prepared by partial acid hydrolysis of chitosan according to Lillo *et al*. (2008). Precipitations were washed and freeze dried. Chitooligosaccharide (1 g) was dissolved in acetic acid to obtain a 3% (wt/vol) solution. Thirty-five ml of 1% TPP solution was added to chitoolgosaccharide solution and stirred for 1 h. Iron sulfate solution 1% was added and pH was fixed at 4.58. The solution was stirred for another 1 h, and centrifuged at 5000 ×g and 4 ˚C. Precipitations were purified and freeze-dried to obtain Fe-CNP.

A total of 480 1-d-old Ross broiler chickens were randomly assigned into four groups with six replicates, 20 birds per replicate. To induce BCO in experimental flock, chickens were reared on wire flooring system (Wideman *et al*. 2012) from one to 42-d-old. Control group was fed with the basal diet formulated to meet or exceed Aviagen requirements for all nutrients without iron supplement. Other treatments included 10 mg/kg Fe from Fe-CNP, 20 mg/kg Fe from $FeSO_4$ and 20 mg/kg Fe from $FeSO_4$ + CNP. The ingredients and chemical composition of diets were shown in Table 1. Production performance including average daily gain (ADG) and feed intake were recorded weekly. Feed conversion ratio (FCR) and performance efficiency factor (PEF) were calculated for each growth periods via the followed formula:

$$FCR = FI_r / WG$$

Where:
FI: feed intake (per bird per period).
WG: weight gain (per bird per period).

$$PEF = (ALW \times SR / D \times FCR) \times 100$$

Where:
ALW: average live weight (kg).
SR: survival rate (%).
D: flock's age (day).
FCR: feed conversion ratio.

At day 39, 10 birds per pen were randomly selected and gait scores (GS) were used to assess their walking ability according to Kestin *et al*. (1992). Firstly, broilers were evaluated macroscopically and divided in two categories: lame or clinically health. Lame birds were marked using a blue color. Birds were placed in a separate pen and walking ability was monitored by three distinct viewers. Each bird numbered from 0 to 5 according to scoring criteria (Kestin *et al*. 1992).

Scores ≥ 3 in treatments considered as impaired gait and lameness. At day 42, blood samples of three birds per pen collected from wing's vein in heparinized tubes. Selected broilers were slaughtered. The thymus, spleen and bursa of fabricius were removed and weighted.

Table 1 Composition of the basal diet

Ingredients	Starter (1-10 d)	Grower (11-24 d)	Finisher (25-42 d)
Corn	54.98	60	61.51
Soybean meal (44%)	37.85	33.14	30.99
Soy oil	2.69	2.7	3.87
Di-calcium phosphate	1.86	1.62	1.45
Oyster shell	1.13	1.04	0.97
Common Salt	0.35	0.3	0.27
Vitamin supplement*	0.25	0.25	0.25
Trace mineral supplement**	0.25	0.25	0.25
DL-methionine	0.34	0.33	0.24
L-lysine	0.34	0.33	0.24
$NaHCO_3$	-	0.07	0.06
Calculated analysis			
Apparent metaboilizable energy (AME) (kcal/kg)	2950	3000	3100
Crude protein (CP %)	22	20	18.89
Met＋Cys (%)	1.03	0.83	0.81
Lys (%)	1.40	1.10	1.04
Met (%)	0.76	0.54	0.52
Ca (%)	0.94	0.84	0.77
Available phosphorous (%)	0.47	0.42	0.38

* Supplied the following per kilogram of diet: vitamin A (trans-retinyl acetate): 3600 IU; vitamin D_3 (cholecalciferol): 800 IU; vitamin E (DL-tocopheryl acetate): 7.2 mg; vitamin K_3: 1.6 mg; vitamin B_1: 0.72 mg; vitamin B_2: 3.3 mg; vitamin B_3: 0.4 mg; vitamin B_6: 1.2 mg; vitamin B_{12}: 0.6 mg; Folic acid: 0.5 mg and Choline chloride: 200 mg.

** Supplied the following per kilogram of diet: Mn (from $MnSO_4 \cdot H_2O$), 40 mg; Zn (from ZnO), 40 mg; Cu (from $CuSO4 \cdot 5H_2O$), 4 mg; I (from Ca $(IO_3)2H_2O$): 0.64 mg and Se (from sodium selenite): 0.08 mg.

The weights were adjusted to one kg live weight and treatment means were calculated. Tumor necrosis-α factor (TNF-α) and Immunoglobulin G (IgG) were assessed by ELISA assay using chicken specific commercial kits (CUSABIO BIOTECH CO., LTD, China) according to manufacturer instruction. The proximal femura and tibia of both legs of broilers were evaluated and diagnosed as follow: macroscopically normal femura and tibia (NF, NT), femoral head separation (FHS), femoral head transitional (FHT) degeneration, femoral head necrosis (FHN), tibial head necrosis (THN). All F= FHS + FHT + FHN (total femoral head lesions). Data were compared by SAS (2004) in a completely randomized design. The statistical model for performance data was $Y_{ij}= \mu + T_i + e_{ij}$. The statistical model for blood and carcass data was $Y_{ijk}= \mu + T_i + e_{ij} + \varepsilon_{ijk}$. The prevalence of abnormal gait (gait score≥3) and diagnostic categories of femora and tibia lesions were analyzed using the X^2 -test.

RESULTS AND DISCUSSION

Growth performance

Table 2 indicates the growth performance of broilers fed different dietary treatments. Iron-deficient diet reduced body weight gain (BWG) and performance efficiency factor (PEF) of broilers in all growth periods (P<0.05). But FI didn't show a stable trend in different periods. It has been suggested that feed efficiency in control group affected by iron deficiency rather than feed intake.

Fe-CNP and $FeSO_4$ hadn't any significant difference in terms of BWG, FCR and PEF in all growth periods. Feeding different forms of iron (i.e. chelate, ferrous sulfate and ferrous sulfate together with chelator agent) didn't show any significant difference in growth performance during 1-42 days (Table 2).

Immunological characteristics

The effects of different treatments on humoral immunity and immune organ indexes are shown in Table 3. Feeding iron with chitooligosaccharide nanoparticles (both chelated or separately) resulted in higher level of IgG in comparison with other groups (P<0.05). Moreover, serum level of TNF-α was significantly decreased in Fe-CNP fed group (P<0.05).

Bursa of fabricus and spleen indexes in broilers receiving dietary supplementation with Fe-CNP and $FeSO_4$ + CNP were increased compared with control and $FeSO_4$ groups. Thymus gland index showed a significant increase in Fe-CNP treatment (P<0.05).

Gait score

Table 4 depicts the percentage of the birds with abnormal gait (gait score≥3) in lame and live (lame or clinically health) birds at 42 d of age. Impaired gait was lower in control and Fe-CNP groups (P=0.053). But in live broilers (both health and lame) there was no difference in the abnormal scores (gait scores≥3) between treatment fed $FeSO_4$ + CNP with three other treatments.

Table 2 The effects of dietary treatments on growth performance

Treatments	Starter (1-10 d)				Grower (11-24 d)				Finisher (25-42 d)				Total (1-42 d)			
	BWG[1]	FI	FCR	PEF	BWG	FI	FCR	PEF	BWG	FI	FCR	PEF	BWG	FI	FCR	PEF
Control	296.78^{b}	332.79^{ab}	1.12^{a}	273.07^{b}	703.69^{b}	1114.59	1.58^{a}	497.31^{b}	1224.67^{b}	2261.42^{b}	1.84^{a}	288.13^{b}	2225.14^{b}	3908.80^{b}	1.75^{a}	302.853^{b}
$FeSO_4$*	335.65^{a}	355.55^{a}	1.05^{b}	327.09^{a}	839.33^{a}	1170.20	1.39^{b}	659.08^{a}	1367.48^{a}	2406.74^{a}	1.75^{b}	337.08^{a}	2542.46^{a}	4132.48^{a}	1.62^{b}	365.040^{a}
Fe-CNP**	329.55^{a}	335.222^{ab}	1.01^{b}	329.55^{a}	8757.70^{a}	1165.72	1.33^{b}	711.25^{a}	1350.21^{a}	2335.94^{ab}	1.72^{b}	353.12^{a}	2555.46^{a}	4186.87^{a}	1.63^{b}	372.627^{a}
$FeSO_4$ + CNP***	316.72^{a}	329.01^{b}	1.04^{b}	317.06^{a}	870.86^{a}	1161.83	1.33^{b}	678.91^{a}	1333.68^{a}	2282.76^{b}	1.71^{b}	343.24^{a}	2521.26^{a}	4123.60^{a}	1.63^{b}	359.045^{a}
SEM	6.4112	7.5071	0.0155	8.5570	22.342	251.1485	0.0232	19.24	18.22	37.303	0.0192	5.6792	29.3296	54.4118	0.001	5.074
P-value	0.0019	0.0894	0.0069	0.0004	< 0.001	0.3882	< 0.001	< 0.001	< 0.001	0.0541	0.004	< 0.001	< 0.001	0.0089	< 0.001	< 0.001

* $FeSO_4$: 20 mg/kg iron types of iron sulfate.
** Fe-CNP: 10 mg/kg iron types of Fe-chitooligosaccharide nanoparticles.
*** CNP + $FeSO_4$: 20 mg/kg iron types of iron sulfate + chitooligosaccharide nanoparticles.
BWG: body weight gain (g); FI: feed intake (g); FCR: feed conversion ratio and PEF: performance efficiency factor.

Table 3 Effect of dietary treatments on immune response of broiler chickens

Treatments	Control	$FeSO_4$*	Fe-CNP**	$FeSO_4$ + CNP***	SEM	P-value
Humoral parameters						
TNF-α (pg/mL)	83.07^{b}	81.662^{b}	87.62^{a}	84.158^{ab}	1.21	0.0004
IgG (µg/dL)	28.41^{c}	29.02^{c}	35.60^{a}	32.63^{b}	0.8917	< 0.0001
Lymphoid organs ratio						
Bursa (%)	1.64^{c}	1.91^{b}	2.11^{a}	2.08^{a}	0.0461	< 0.0001
Thymus (%)	3.44^{c}	4.07^{bc}	4.77^{a}	4.37^{ab}	0.1567	0.0051
Spleen (%)	1.15^{c}	1.46^{b}	1.80^{a}	1.82^{a}	0.0717	< 0.0001

* $FeSO_4$: 20 mg/kg iron types of iron sulfate.
** Fe-CNP: 10 mg/kg iron types of Fe-chitooligosaccharide nanoparticles.
*** CNP + $FeSO_4$: 20 mg/kg iron types of iron sulfate + chitooligosaccharide nanoparticles.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 4 Percentage of impaired gait scores (GS≥3) in lame and live (both health and lame) broilers

Item	Control	$FeSO_4$*	Fe-CNP**	$FeSO_4$ + CNP***	SEM	P-value
Lame broilers						
GS ≥ 3	33.33^{b}	56.67^{a}	30^{b}	46.67^{ab}	7.071	0.053
Live broilers						
GS ≥ 3	16.66	26.66	23.33	30	5.322	0.355

* $FeSO_4$: 20 mg/kg iron types of iron sulfate.
** Fe-CNP: 10 mg/kg iron types of Fe-chitooligosaccharide nanoparticles.
*** CNP + $FeSO_4$: 20 mg/kg iron types of iron sulfate + chitooligosaccharide nanoparticles.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Leg evaluation

Table 5 illustrates the percentage of evaluated legs of live broilers at the end of experiment (42 d). Diagnostic categories of BCO lesions significantly affected by dietary treatments (P<0.05). Live broilers in Fe-CNP and control groups exhibited a higher incidence of normal femora and a lower percentage of transitional femur necrosis (FHT) and total femur lesions (All F; P<0.05). Whereas, FHN and FHS were not affected significantly by dietary treatments (P>0.05).

In the current study, the control diet had Fe content below the Aviagen specifications for broilers. Therefore, it depressed growth performance and affected FCR. Totally, in terms of growth performance and efficiency, there were no significant difference between two Fe sources at levels of usage. Results showed that 10 mg/kg Fe in the form of Fe-CNP had the same efficiency as 20 mg/kg Fe in the form of $FeSO_4$. In some researches, supplemental organic minerals have revealed better efficiency than inorganic sources (Bao *et al*. 2007; Nollet *et al*. 2007).

The use of nano scale minerals in poultry feeding has shown some beneficial effects on growth performance (Ahmadi *et al*. 2018; Asheer *et al*. 2018), immune response (Shirsat *et al*. 2016) and bioavailability compared with common mineral supplements (Asheer *et al*. 2018), which partially are in accordance with our results. Also, lower toxicity of metal nanoparticles makes them more efficient than salts of the same metals (Kovalenko and Folmanis, 2006). In the current experiment, there is no difference in growth performance between dietary nano iron and iron sulfate groups, which probably is related to the level of usage.

Chitooligosaccharide nanoparticles which were used in current study as iron chelator agent is a chitosan derivate. Because of mucoadhesive properties of chitosan, it could be useful in drug delivery systems (Lehr *et al*. 1992) and increase absorption rate of loaded material.

The effect of iron on immune system is probably due to its role in secretion of TNF-α and interleukins (Safuanova *et al*. 2004).

Table 5 Percentage of diagnostic categories of bacterial chondronecrosis with osteomyelitis (BCO) lesions in evaluated legs of live broilers in day 42 (different categories were analyzed between treatments)

Treatments	NF	FHT	FHN	FHS	All F	NT	THN
Control	56.25[a]	14.583[b]	10.41	20.83	45.833[b]	41.67[ab]	58.33[ab]
$FeSO_4$*	35.417[b]	31.25[a]	14.58	20.833	66.66[a]	25[b]	75[a]
Fe-CNP**	66.66[a]	12.5[b]	6.25	16.66	35.41[b]	66.67[a]	33.33[b]
$FeSO_4$ + CNP***	39.583[b]	27.08[a]	14.58	22.91	64.58[a]	33.33[b]	66.67[a]
SEM	4.36	3.81	3.95	3.75	6.07	10.33	10.33
P-value	0.0003	0.0049	0.4053	0.6950	0.0039	0.0531	0.0531

* $FeSO_4$: 20 mg/kg iron types of iron sulfate.
** Fe-CNP: 10 mg/kg iron types of Fe-chitooligosaccharide nanoparticles.
*** CNP + $FeSO_4$: 20 mg/kg iron types of iron sulfate + chitooligosaccharide nanoparticles.
Evaluated legs of live broilers at the end of experiment were assigned into one of the diagnostic categories of BCO lesions as follow: NF: normal femora; FHS: femoral head separation; FHT: femoral head transitional; FHN: femoral head necrosis; NT: normal tibiae and THN: tibial head necrosis. All F= FHS + FHT + FHN (total femoral head lesions).
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

In current study iron deficient diet increased TNF-α and decreased IgG levels. It is demonstrated that iron deficient status causes cellular (Kuvibidila and Porretta, 2003; Safuanova *et al*. 2004) and humoral immunodeficiency (Ekiz *et al*. 2005). It has been reported a decrease in T-cell proliferation (Kuvibidila and Porretta, 2003), and immunoglobulin levels (Ekiz *et al*. 2005) and increase in TNF-α and other pro-inflammatory cytokines (Jason *et al*. 2001; Safuanova *et al*. 2004) in iron deficient status.

In other hand, it has been shown that oral iron chelators inhibit NF-κB (Banerjee *et al*. 2015), which is the master regulator of deregulated cytokines such as TNF-α (Messa *et al*. 2010; Cheng *et al*. 2011; Ruan *et al*. 2011). In fact, NF-κB accounts for the link between excessive iron and oxidative stress. Iron supplementation by activating NF-κB, induces inflammation.

In current study TNF-α reduction in Fe-CNP treatment is probably due to reducing of free iron and subsequently oxidative stress. Overall both iron deficiency (as in control treatment) and excessive free iron (as in $FeSO_4$) increased TNF-α level. TNF-α is produced by macrophages and T-cells and is shown to induces tissue injureis (Pasparakis *et al*. 1996). It accounts for an intestinal inflammation and permeability marker and determines the immune cell response (Alhenakya *et al*. 2017). We speculated that iron chelating affects BCO occurrence by two mechnaysms: 1- by improving intestinal permeability (reflected by lower serumic TNF-α), 2- by improving immune system function (reflected by the increase of IgG).

Disturbance in intestinal permeability defines as disturbance of selective intestinal transport (e.g., toxin and viable bacteria) (Troeger *et al*. 2009) and weakening of tight junctions. Altering the permeability of paracellular pathways facilitate luminal bacteria and their products to translocate from the intestinal lumen to other organs. Bacterial translocation is the key step in pathogenesis of BCO.

The translocated bacteria reach to the zone of necrosis and adhere to the cartilage matrix in osteochondotic clefts and reveal BCO signs. As it is evident, intestinal permeability has been reduced in Fe-CNP which reflected in significantly lower TNF-α. In accordance with current results, Ghazi *et al*. (2012) have reported the higher IgG titer in broiler chickens fed with organic minerals compared with inorganic minerals. On the other hand, Haghighi *et al*. (2006) have reported that a higher IgG concentration is related to colonization of commensal bacteria in the intestine.

Therefore, the higher IgG concentration in Fe-CNP might be associated with intestine commensal bacteria such as *Lactobacillus* and *Bifidobacterium*. These observations demonstrate the effects of Fe-CNP on intsetinal microflora composion.

In current study bursa of Fabricius and spleen relative weights were significantly increased in Fe-CNP and $FeSO_4$ treatments ($P<0.05$). But only in Fe-CNP there is an improvement in thymus gland relative weight. Lymphoied organs are involved in cellular immune responses. Bursa is the primary site of B cell development, which produces immunoglobulins (Pike *et al*. 2004). In control treatment malnutrition of iron resulted in lymphoied organs atrophy. Feeding of Fe-CNP and $FeSO_4$ + CNP resulted in higher serum IgG concentration accompanied by improving lymphoied organs relative weights. Improvement of lymphoied organs relative weights in these two treatments might be the result of immune modulating effects of chitosan (or its derivates) which has been reported previously by Shi *et al*. (2005). Since in $FeSO_4$ + CNP didn't reduce the percentage of necrotic femur and tibia (FHN and THN respectively) it is probable that cellular and humoral immunity may not participate in reducing BCO lesions. These observations are in accordance with other researches who demonstrated that both cellular and antibodies of immune system are not capable to

sequestrating bacterial foci in bone head lesions (Smeltzer and Gillaspy, 2000; Kense and Landman, 2011).

Dietary treatments had significant effects on diagonistic categories of live birds in day 42 (Figure 1). Totally $FeSO_4$ increased femur and tibia lesions both separately or when fed with CNP (non-chelated form). Iron chelating reduced femur and tibia lesions, but not FHN and FHS lesions. Fe-CNP reduced the percentage of all femur lesions which had progressed to FHN.

It was concluded that Fe-CNP effectively delayed the occurrence of severe BCO lesions until slaughter weight. In other words Fe-CNP reduced transformation of FHS lesions into FHT. Control treatment showed a partially similar trend with Fe-CNP for femur lesions. As mentioned above, weight gain was reduced in control in comparison with other treatments. It is probable that the pressure due to high growth rate on epiphyseal-physeal cartilage which is one of the initial causes of BCO pathogenesis has been prevented. But immunesuppresion due to iron defficiency in this treatment (as mentioned earlier) resulted in transformation of generated early BCO lesions into necrosis. It seems that tibia was more sensitive to BCO, which reflected in higher THN percentage than FHN in all treatments. In accordance with this result, Wideman *et al*. (2015) have reported the higher incidence of THN in live and clinically healthy broilers at the end of experiment. Totally iron chelating resulted in a higher percentage of normal femur and tibia in live birds whether clicically healthy or lame. These results are in compability with immune system improvement of Fe-CNP treatment.

Sirri *et al*. (2016) have shown that organic trace minerals containing Cu, Mn and Zn in broiler chicken diet significantly reduced the prevalence of femur and tibia lesions compared with inorganic sources. They suggested that the higher bioavailability of organic mineral sources could increase bone mineralization and affect bone susceptibility to bacterial infection.

In the control group, the improvement in gait score might be related to a reduction in BW. Gait scoring (GS) method proposed by Kestin *et al*. (1992) characterizes the impairment in walking ability mainly due to lameness. Wideman *et al*. (2015) attributed the impair gait score to the infectious conditions. But in some cases there is no detectable signs of gait impairment in broilers with macroscopic BCO lesions. In current study there was no significant difference in terms of impaired gait scores (scores≥3) between dietary treatments in live on day 42. But in lame birds it was evident that the percentage of impaired gait was significantly increased in $FeSO_4$ treatment ($P< 0.05$). It was concluded that gait score may not accurately evaluate skeletal disorders such as BCO as mentioned by previous researches (Naas *et al*. 2009; Sandilands *et al*. 2011).

CONCLUSION

In the current study, iron chelate in the form of Fe-CNP did not affect growth performance and FCR compared with $FeSO_4$, whereas its iron content was 50% lower than $FeSO_4$ treated group. Therefore Fe-CNP: $FeSO_4$ utilization ratio was 100%. The notable effect of Fe-CNP on BCO reflected in reduction of intestine permeability and consequently reduction of femur and tibia lesions in live birds at the end of the experiment.

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