Int. J. Nanosci. Nanotechnol., Vol. 10, No. 3, Sept. 2014, pp. 187-196

Short Communication

Iron Nanoparticles and Methionine Hydroxy Analogue Chelate *in ovo* Feeding of Broiler Chickens

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(Received: 31 Jan. 2014 and accepted: 12 Aug. 2014)

Abstract:
The 440 fertile eggs, in a completely randomized design were divided into 11 treatments with four replicates and 10 eggs per each replicate. Treatments including: (T1; control; without injection), (T2; injected with 0.3 ml saline 9.0%; sham control), (T3, T4 and T5) 25, 75, 125 ppm Fe-Nano, (T6, T7 and T8) 50, 100, 150 ppm Fe-Nano-Alimet chelate, (T9, T10 and T11) 50, 100, 150 ppm Fe-Aliment chelate. On 10th day of incubation, 0.3 ml solution in ovo was injected into the egg yolk sac. Higher hatchability was found in controls, 25 ppm Fe-Nano, 100 ppm Fe-Nano-Alimet chelate and all levels of Fe-Alimet chelate treatments than other treatments. The egg weight was higher significantly in sham control, 25 and 75 ppm Fe-Nano, all levels of Fe-Nano-Alimet chelate and 150 ppm Fe-Aliment chelate treatments. There was significant increase in chick weight in controls, 25 and 75 ppm Fe-Nano, 100 ppm Fe-Nano-Alimet chelate and 150 ppm Fe-Aliment chelate in comparison with other treatments. Also chick body weight to egg weight ratio in controls, 25ppm Fe-Nano and 100ppm Fe-Nano-Alimet chelate was higher than in other treatments. Level of 25 ppm of Fe-Nano has shown the highest relative liver weight. Serum Fe content and liver was higher by using 25 ppm Fe-Nano, 100 ppm Fe-Nano-Alimet chelate and 150 ppm Fe-Aliment chelate than other treatments. By applying two treatments of 100 ppm Fe-Nano-Alimet chelate and 150 ppm Fe-Aliment chelate, chickens blood hemoglobin increased significantly compared with the other treatments. These results suggest that 25 ppm Fe-Nano, 100 ppm Fe-Nano-Alimet chelate and 150 ppm Fe-Alimet chelate improved embryonic growth and development.
Keywords: *Broiler chicken, Chelate, hatchability, In ovo, Iron nanoparticles, Methionine*

1. INTRODUCTION

Iron is essential for animal and poultry and supplemented in their diets. Iron is an integral part of many proteins and enzymes that maintain good health and help oxygen transport [1]. It is also essential for the regulation of cell growth and differentiation [2], the production of hemoglobin, myoglobin and the component of red blood cells that transports oxygen around the body. By activating or assisting enzymes, iron is involved at every stage of the tricarboxylic acid cycle, and iron-containing catalase and peroxidases remove potentially dangerous products of metabolism, while iron-activated hydroxylases influence the connective tissue development [3,4]. Normal embryonic growth and development depends on a complete supply of all required nutrients within the egg. NRC [5] recommended 50-120 ppm of iron for poultry and 2,000 ppm for tolerance limit.

On the other hand, methionine (Met) is sulphur containing amino acid. One commonly used source of Methionine activity is 2-hydroxy-4-(methylthio) butanoic acid (HMTBA) which can be converted to L-Met within the body of the animal through broadly distributed enzymatic systems [6-8]. HMTBA is an organic acid which bears a hydroxyl group on α carbon instead of the amino group found in Methionine. [9].

Chelated or complex trace elements are more efficiently absorbed from the gut than those provided by inorganic salts. Traditionally, the inorganic salts (oxides, sulphates) have been used in poultry diets due to their suitable price and because they meet the requirements [10]. Methionine is the most commonly used amino acid chelating agent [11-14].

Currently, nanoparticle are inorganic or organic particles with diameters ranging from 1 to 100 nm [15]; while there are examples of nanoparticles (NPs) which are several hundreds of nanometers in size. They have many novel properties compared with the bulk materials. There are several areas in which nanotechnology could be applied to the science and engineering of agriculture, animal and food systems [16-18]. Metal nanoparticles are less toxic than salts of the same metals and have an advantage of a prolonged effect on biological objects [19]. Toxicological studies of nanosized iron have shown that such iron powders are low toxic. Iron oxide nanoparticles are inherently biocompatible [20] due to their general stability in air and their ability to be degraded or metabolised in vivo, making them excellent candidates for a large variety of applications [21].

Nutrient administration in ovo may provide an alternative method for poultry companies to increase chick weight at hatch day. Chicks are affected by the nutrients in yolk remaining in the peritoneal cavity post hatching [22]. Data on mineral levels in the egg compartments during incubation are very limited. Furthermore, today's "high-metabolism, fast-growing" broiler embryos and chickens [23] may reach levels of mineral deficiency that can lead to metabolic disorders. Little research has dealt with nanotechnologies in animal nutrition and feed improvement. Thus, the aim of this study is to examine effects of iron, iron nanoparticle and chelates of iron nanoparticles-methionine on hatch parameters (mortality, hatchability, chick weight and chick weight/ egg weight) in chickens.

2. MATERIAL AND METHODS

2.1. Preparation of Materials:

2.1.1. Alimet: This is a liquid product of methionine hydroxy analogue (2-hydroxy- 4-methylthio butanoic acid, HMTBA, Novus International, Inc., Charles, MO, USA.

2.1.2. Iron Nanoparticles: According to Reimers and Khalafalla [24], iron nanoparticles were produced by co-precipitation from an aqueous Fe^{3+}/Fe^{2+} solution (ratio 3:2) using concentrated ammonium hydroxide in excess. 14.6 g of $FeCl_3$ and 12.0 g of $FeCl_2 .4H_2O$ were dissolved in 50 ml distilled water, 40 ml of ammonium hydroxide were added rapidly. The mixture was centrifuged and precipitate was removed. The precipitate was washed for three times with highly purified water to remove the unreacted chemicals, then the product was dried in the vacuum. The brown mixture was then aged at 110°C for 6h to evaporate water and excess of ammonium. The black lump-like residuum was cooled to room temperature and washed several times with distilled water. After drying, a powder was obtained. Iron oxide nanoparticles were identified by different analytical methods.

2.1.3. Iron Alimet Chelate: according to Predieri *et al.* [25], chelate was prepared by reaction of the Alimet with iron (II) sulfate. A pale yellow precipitate was obtained which, after filtration, washed and dried, was recognized as $[\{CH_3SCH_2CH_2$ CH (OH) COO$\}2$ Fe$]2$ H_2O by elemental C, H, S analysis.

2.1.4. Fe-Nano-Alimet Chelate: Based on Marinescu *et al.* [26], to prepare an aqueous solution of a mixture of Fe(III) and Fe(II) ions in a molar ratio (2:1), a solution of amino acid in the molar ratio (2Fe(III):1-Fe(II):2 Alimet, (Alimet was added and kept at constant temperature of 60°C for 15 min under stirring. Ammonia solution (12.5%)

was then added drop wise to the iron-amino acid solution, till a compound was separated (pH=3–4). This suspension was maintained at 600 for 30 min, under vigorous stirring. The fine powder was separated from the aqueous solution by filtration, rinsed with distilled water and alcohol and then dried on P4O10 at room temperature.

X-ray diffraction (XRD) pattern of dry sediments was determined by (APD2000) X-ray diffractometer (Cu Kαradiation, λ= 1.54176 Å,) using a continuous scan mode, and 2θ data were collected from 2 to 99, with 15 s counting per 0.01° 2θ step. FT-IR spectra for iron nanoparticles were recorded in the transmission mode on a FT-IR Spectrophotometer. To study the morphology and chemistry of iron nanoparticles and iron nanoparticles methionine chelate, the TEM was performed using the EM208 (Philips Holland) at a voltage of 100 Kv, and the CM120 (Philips Holland).

2.2. Incubation and in ovo Feeding (IOF):

A Total of 440 fertile eggs of (Ross-308) broiler breeder strain at 30 weeks of age were obtained from Dostan-Hamedan hatchery and incubated according to standard hatchery practices in laboratory, Bu-Ali Sina University. Eggs were incubated at 37.7°C, 60% humidity and automatically turned every hour. On 10th day of incubation, each egg was candled.

Then, eggs were individually weighed with normal distribution of 59.34 g. These eggs were evenly assigned to eleven treatments and 4 replicates with 10 eggs in each. A hole was incised using a dental drill and 0.3 ml of IOF solution was injected into the yolk sac using a 22-gauge needle to a depth of about 13 mm. The injection site was disinfected with ethyl alcohol, sealed with wax, and transferred to hatching baskets.

The IOF solutions (iron nanoparticles (Fe-Nano), Fe-Nano-Alimet chelate and Fe-Alimet chelate contained the following: (1) control (without injection), (2) injected with 0.3 ml saline 9.0% (sham control), (3) 25 ppm Fe-Nano, (4) 75 ppm Fe-Nano, (5) 125 ppm Fe-Nano, (6) 50 ppm Fe-Nano-Alimet chelate, (7) 100 ppm Fe-Nano-Alimet chelate, (8) 150 ppm Fe-Nano-Alimet chelate, (9) 50 ppm Fe-Alimet chelate, (10) 100 ppm Fe-Alimet chelate, 11) 150 ppm Fe-Alimet chelate. Fertility was determined by candling after first week of incubation. Embryonic mortality (%) was calculated after break open of non-hatch eggs and recorded embryonic mortalities as follows: (Number of embryo mortality / Number of fertile on the day of hatch eggs) × 100. (d 21 of incubation), chicks were weighed individually.

Hatchability was determined at the end of incubation. Hatchability of fertile eggs was found via a similar way in which the number of live chicks was divided by the number of fertile eggs kept in the incubator [24].

The carcasses were detached and different parts of them such as breast meat, leg meat and liver were weighted by digital balance with 0.01 g accuracy. Blood samples were collected for hemoglobin analysis. Liver samples were thoroughly cleaned in running tap water and dried according to an AOAC method [28] after wet ashing with HNO3 and HCl, iron was measured. Fe was measured by flame atomic absorption spectrometry (GF-AAS, Perkin Elmer 3030).

Blood samples were collected into heparinized tubes for Hemoglobin (Hb) and iron concentrations. Samples were centrifuged at 3000 rpm for 20 min and plasma was separated and kept in the temperature -20°C followed by defrosting and testing by spectrophotometer model UNICO 2100 Vis (South Korea). Hemoglobin (Hb) concentration was measured calorimetrically, using a diagnostic kit according to the manufacturer instructions. Iron contents were measured using inductively coupled plasma spectrometry.

2.3. Statistical Analyses

A completely randomized design was applied. Data were analyzed by the GLM procedure (SAS Institute 2004). Duncan's multiple range tests was used for comparison of meanings ($P<0.05$).

3. RESULTS

The XRD scattering measurement of the powdered sample provided structural information through

the determination of crystallinity. With the XRD pattern of iron nanoparticles and Fe-Nano-Alimet chelate, the average diameters were evaluated as 8-12 nm and 14-22nm (Figure1).

The FT-IR spectrum of Fe-Nano-Alimet chelate has shown diffraction peaks at (799), (892), which are the characteristic peak of the Fe-Nano (Figure2). Because the carboxyl groups were potential coordination sites for the metal ions, the changes in the FT-IR absorption peaks in the C=O stretching frequency were monitored as evidence of metal complex formation.

Figure 3 shows TEM photograph size and shape of Fe-Nano and Fe-Nano-Alimet chelate prepared under standard conditions. It is worthwhile to note that the size distributions are 8-12 nm and 14-22 nm and the Fe-Nano is spherical in shaped, uniform and mono dispersed.

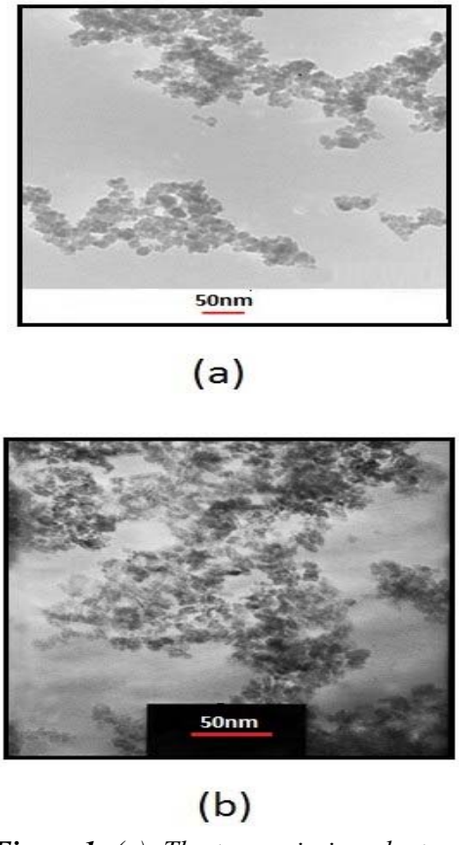

Figure 1: *(a), The transmission electron microscope (TEM) image of Fe-Nano. (b), The transmission electron microscope (TEM) image of Fe-Nano-Alimet Chelate.*

Percentages of fertility, embryonic mortality and hatchability data are showed in Table 1. Results indicate that the differences between percentages of fertility were not significant among the experimental groups. The lowest mortality rate was observed for 50 and 150 ppm Fe-Alimet chelate, control 1 (without injection), 25 ppm Fe-Nano and 100 ppm Fe-Nano-Alimet chelate. Hatchability was higher in controls, 25 ppm Fe-Nano, 100ppm Fe-Nano-Alimet chelate and all levels of Fe-Alimet chelate treatments compared with other treatments.

The data of in ovo injection of Fe-Nano, Fe-Nano-Alimet chelate and Fe-Alimet chelate on initial egg weight, chick body weight and Chick weight/ egg weight in hatch day are shown in Table 2. The egg weight was significantly higher in sham control, 25 and 75 ppm Fe-Nano, all treatments of Fe-Nano-Alimet chelates and 150 ppm Fe-Alimet chelate treatments.

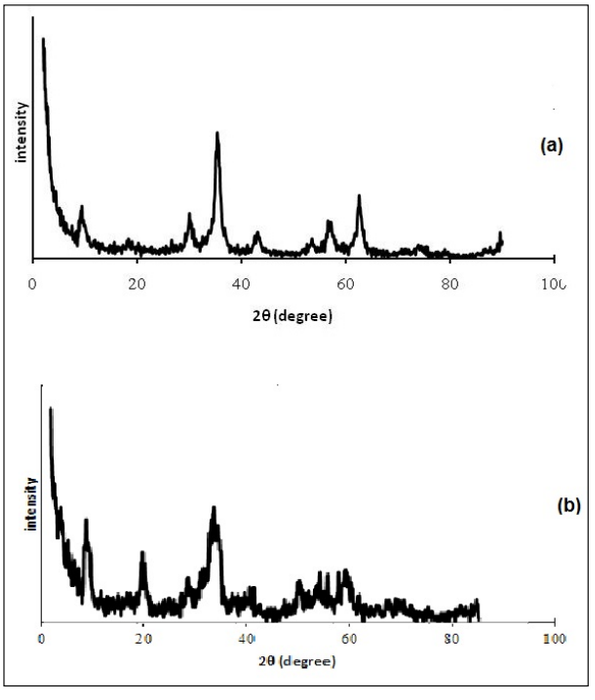

Figure 2: *(a), XRD of Fe-Nano. (b), XRD of Fe-Nano-Alimet Chelate.*

In this experiment there was a significant increase in chicks' initial body weight in control treatments, 25 and 75 ppm Fe-Nano, 100 ppm Fe-Nano-Alimet

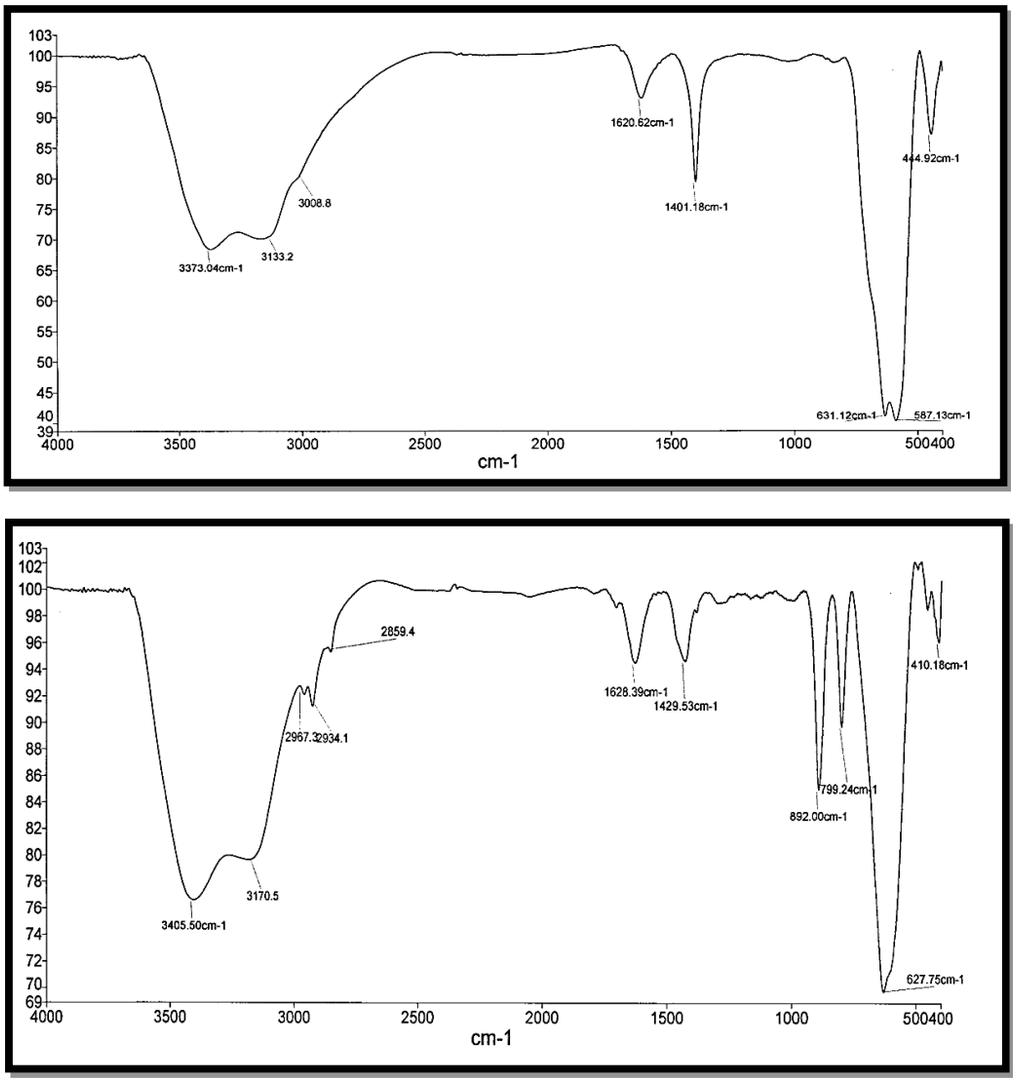

Figure 3: *(a), FTIR of Fe-Nano. (b), FTIR of Fe-Nano-Alimet Chelate.*

chelate and 150 ppm Fe-Alimet chelate treatments. Also chicks' body weight to egg weight ratio in control treatments, 25 ppm Fe-Nano and 100ppm Fe-Nano-Alimet chelate was higher compared with other treatments.

The data of in-ovo feeding on carcass hatchability in Table 3 shows that highest liver weight was related to the treatment having 25 ppm of Fe-Nano. Heart, breast, and leg relative weight and breast weight to body weight ratio were not significantly different among all treatments.

The data of in ovo injection of Fe-Nano, Fe-Nano-Alimet chelate and Fe-Alimet chelate and effects on Fe in liver and blood parameters of broiler in hatch day are presented in Table 4. The treatments containing 25 ppm Fe-Nano, 100 ppm Fe-Nano-Alimet chelate and 150 ppm Fe-Alimet chelate have shown higher Fe content in serum and liver compared with other treatments. Also, in two treatments of 100 ppm Fe-Nano-Alimet chelate and 150 ppm Fe-Alimet chelate hemoglobin significantly increased compared with other treatments.

Table 1: *Effect of in ovo injection on Fertility percentage, mortality and hatchability in hatch day (%)*

Treatments	Fertility	Mortality	Hatchability
Non-Injected Control	70.46	0.5^{d}	93.75^{a}
Sham* Control	68.19	1.0^{dc}	86.61^{ab}
25 ppm (Fe-Nano)	61.37	1.5^{abcd}	78.27^{abcde}
75 ppm (Fe-Nano)	61.37	2.3^{abc}	66.07^{cde}
125 ppm (Fe-Nano)	68.19	2.0^{abc}	72.77^{bcde}
50 ppm (Fe-Nano-Alimet chelate)	63.64	2.8^{a}	60.42^{e}
100 ppm (Fe-Nano-Alimet chelate)	68.19	1.3^{bcd}	83.04^{abcd}
150 ppm (Fe-Nano-Alimet chelate)	65.91	2.5^{ab}	64.73^{de}
50 ppm (Fe-Alimet chelate)	72.73	0.3^{d}	96.88^{a}
100ppm (Fe-Alimet chelate)	68.19	1.0^{dc}	85.42^{abc}
150ppm (Fe-Alimet chelate)	70.46	0.5^{d}	93.75^{a}
SEM	3.28	0.42	6.13
P-value	0.92	0.03	0.009

** Sham, injected with 0.3 ml of NaCl 0/9%. Means with common superscripts in the same column are not significantly different (P < 0.05). SEM: standard error of the means.*

Table 2: *The effect of in ovo injection on initial egg weight, chick body weight and Chick weight/ egg weight in hatch day*

Treatments	Egg weight (g)	Chick weight (g)	Chick weight/ egg weight
Non-Injected Control	59.40^{bc}	41.10^{ab}	69.19^{a}
Sham* Control	61.13^{abc}	41.03^{ab}	67.01^{abc}
25 ppm (Fe-Nano)	61.75^{ab}	40.50^{abc}	65.60^{abcde}
75 ppm (Fe-Nano)	61.43^{abc}	40.10^{abc}	65.27^{bcde}
125 ppm (Fe-Nano)	59.73^{bc}	36.95^{d}	61.87^{e}
50 ppm (Fe-Nano-Alimet chelate)	60.88^{abc}	38.80^{bcd}	63.75^{cde}
100 ppm (Fe-Nano-Alimet chelate)	62.55^{a}	42.65^{a}	68.21^{ab}
150 ppm (Fe-Nano-Alimet chelate)	60.98^{abc}	38.30^{cd}	62.81^{de}
50 ppm (Fe-Alimet chelate)	58.70^{c}	37.98^{cd}	64.84^{bcde}
100ppm (Fe-Alimet chelate)	59.03^{bc}	37.95^{cd}	64.23^{cde}
150ppm (Fe-Alimet chelate)	62.74^{a}	41.50^{a}	66.18^{abcd}
SEM	4.21	0.813	1.18
P-value	0.039	0.008	0.007

**Sham, injected with 0.3 ml of NaCl 0/9%. Means with common superscripts in the same column are not significantly different (P < 0.05). SEM: standard error of the means.*

4. DISCUSSION

The XRD pattern evaluated the average diameter of iron nanoparticles and Fe-Nano-Alimet chelate 8-12 nm and 14-22nm, respectively. Nikoniv *et al.* [29] obtained that at least 75% of the sizes of iron oxide nano particles was in the range from 7.5 to 20 nm.

Also they studied the possibility of using iron nanoparticles as micronutrient components of feed for poultry. Kinoshita *et al.* [30] carried out a study on the magnetic separation of amino acids by Au/ Fe oxide composite nanoparticles. They found that among 17 amino acids used in this experiment only two sulfur-containing amino acids (cystine and methionine) were adsorbed onto the gold-coated

Table 3: *The effect of in ovo injection on organs relative weight in hatch day*

Treatments	Liver[2]	Heart[2]	Breast[2]	Leg[2]	Breast W/ Body W
Non-Injected Control	1.95^{bcde}	0.44	0.77	0.37	1.88
Sham[1] Control	1.97^{bcde}	0.40	0.82	0.35	1.98
25 ppm (Fe-Nano)	2.44^{a}	0.46	0.81	0.38	2.00
75 ppm (Fe-Nano)	1.95^{bcde}	0.38	0.74	0.29	1.85
125 ppm (Fe-Nano)	1.76^{e}	0.43	0.73	0.33	1.98
50 ppm (Fe-Nano-Alimet chelate)	2.05^{bcd}	0.40	0.82	0.35	2.11
100 ppm (Fe-Nano-Alimetchelate)	2.18^{b}	0.43	0.87	0.45	2.05
150 ppm (Fe-Nano-Alimet chelate)	1.86^{cde}	0.38	0.78	0.36	2.04
50 ppm (Fe-Alimet chelate)	2.07^{bcd}	0.45	0.74	0.28	1.94
100ppm (Fe-Alimet chelate)	1.83^{de}	0.41	0.79	0.36	2.09
150ppm (Fe-Alimet chelate)	2.13^{bc}	0.51	0.09	0.38	2.18
SEM	0.084	0.025	0.042	0.038	0.117
P-value	0.001	0.064	0.24	0.32	0.84

1 Sham, injected with 0.3 ml of NaCl 0/9%. Means with common superscripts in same the column are not significantly different (P < 0.05). SEM: standard error of the means. 2 (% of live body weight).

Table 4: *The effect of in ovo injection on liver and blood Fe concentration of chicken in hatch day*

Treatments	liver Fe (µg/g)	blood Fe (g/dl)	Hemoglobin (g/dl)
Non-Injected Control	33.55^{cd}	97.52^{c}	8.85^{c}
Sham[1] Control	37.94^{bcd}	99.10^{c}	8.85^{c}
25 ppm (Fe-Nano)	41.58^{abc}	107.46^{abc}	10.06^{bc}
75 ppm (Fe-Nano)	39.10^{bcd}	102.41^{bc}	9.87^{bc}
125 ppm (Fe-Nano)	38.83^{bcd}	101.94^{bc}	9.68^{bc}
50 ppm (Fe-Nano-Alimetchelate)	37.27^{bcd}	102.53^{bc}	9.17^{c}
100 ppm (Fe-Nano-Alimet chelate)	42.45^{ab}	114.98^{a}	11.50^{ab}
150 ppm (Fe-Nano-Alimet chelate)	40.19^{bc}	100.00^{bc}	9.32^{c}
50 ppm (Fe-Alimet chelate)	31.15^{d}	101.22^{bc}	9.97^{bc}
100ppm (Fe-Alimet chelate)	41.33^{abc}	101.47^{bc}	9.89^{bc}
150ppm (Fe-Alimet chelate)	45.50^{a}	109.57^{ab}	12.13^{a}
SEM	2.58	2.99	0.559
P-value	0.014	0.019	0.014

1Sham, injected with 0.3 ml of NaCl 0/9%. Means with common superscripts in the same column are not significantly different (P < 0.05). SEM: standard error of the means.

Fe oxide nanoparticles.

A variety of trace elements including zinc, copper and iron are required in differing amounts to sustain proper growth and development of the avian embryo [31]. Deficiencies or excesses of individual trace elements can cause impaired growth, abnormal development affecting all of the major organ systems and, in extreme cases, death of the embryo [32]. Thus, a continually and precisely regulated supply of trace elements derived from stores within the egg is essential to ensure avian embryonic survival. Appropriate amounts of each trace element are required to support embryonic growth and development [33].

As our results indicated the hatchability was higher in controls, 25 ppm Fe-Nano, 100 ppm Fe-Nano-

Alimet chelate and all levels of Fe-Alimet chelate treatments. Similarly, researchers [34] found a significant reduction in early embryo mortality when breeder hens were fed a Zn-amino acid complex as the only source of Zn supplementation. Also have been reported 3.6 more chicks per hen housed in hens consuming diets supplemented with a racemic mixture of zinc sulfate and a Zn-amino acid complex compared with hens consuming only the diet supplemented with the Zn-amino acid complex [34].

Kidd *et al.* found an increase in fertility in hens fed a diet supplemented with a Zn-amino acid complex compared with a control diet (without Zn supplementation); however, only a numerical response for hatchability was observed [35].

Also chicks' body weight to egg weight ratio in control treatments, 25 ppm Fe-Nano and 100 ppm Fe-Nano-Alimet chelate treatments was higher compared with those in other treatments. Fe linking to an amino acid increased the transfer of Fe across the placenta and into the embryo Ashmead and Graff [36].

Also, an amino acid solution injected into the eggs on the 7th day of incubation improved body weights relative to egg weight [37]. Experiments revealed that in ovo feeding carbohydrates, (β-hydroxy-β-methylbutyrate) HMB, or both carbohydrates and HMB [37] increased broiler body weight, relative pectoralis breast muscle. Also in this experiment there was a significant increase in chicks' initial body weight in control treatments, 25 and 75 ppm Fe-Nano, 100 ppm Fe-Nano-Alimet chelate and 150 ppm Fe-Alimet chelate treatments.

The transport of iron from enterocytes to blood depends on the iron pool in the liver. A high level of iron results in the synthesis of large amounts of hepcidin in liver. In this research breast weight (%) was not significantly different among all treatments. The pectoral or breast muscle of the avian embryo is metabolically important, mainly because of its relatively large size and glycogen storage capacity. Even though the pectoral muscle contains less glycogen per unit of mass than the liver, it accounts for the greatest quantity of total glycogen stored in the body [38-39].

The pectoral muscle is also the predominant source of protein mobilized to supply amino acids for gluconeogenesis if energy reserves are depleted after hatch [40].

The results indicated that the absolute amount of iron per liver increased steadily up to hatching time. As results of this article show the highest liver weight was observed in treatment having 25 ppm of Fe-Nano. The treatments 25 ppm Fe-Nano, 100 ppm Fe-Nano-Alimet chelate and 150 ppm Fe-Alimet chelate have shown higher Fe content in serum and liver compared with those in other treatments. Seo *et al.* [41] concluded that iron content of broiler meat can be effectively enriched by supplementation of 200 ppm of Fe as Fe-Alimet chelate for 5 weeks. The results was observed for iron concentrations in the liver and kidney of pigs [42-43] and chickens for fattening [41, 44]. The greatest mean increase was +22% and +31.9% for broiler muscle and liver, respectively.

Also, hemoglobin in two treatments of 100 ppm Fe-Nano-Alimet chelate and 150 ppm Fe-Alimet chelate significantly increased compared with other treatments.

5. CONCLUSION

These results suggest that 25 ppm iron nanoparticles (Fe-Nano), 100 ppm iron nanoparticles Alimet chelate (Fe-Nano-Alimet chelate) and 150 ppm Fe-Alimet chelate as injection contributed to embryonic growth development. Iron nanoparticles and Alimet chelate form, as the active ingredient of feed additives, premixes, and compound feed, due to the high surface activity and penetration into cells can actively influence the intracellular metabolism by stimulating various processes.

ACKNOWLEDGMENT

We specially thank Bu-Ali Sina University for providing the facilities and the financial support in this study. We also thank the staff of the Department of Animal Science and Faculty of Chemistry in this university for their excellent scientific collaboration.

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