Broiler intestine *DMT1* gene expression and bone characteristics, as affected by *in ovo* injection of different forms of manganese

Minoo Mirzavandi Chegenia, Majid Mottaghitala, Seyed Hossein Hosseini Moghaddama and Mostafa Golshekan

aDepartment of Animal Science, Faculty of Agriculture Science, University of Guilan, Rasht, Iran; bInstitute of Medical Advanced Technologies, Guilan University of Medical Sciences, Rasht, Iran

**ABSTRACT**

This study was conducted to compare the effects of *in ovo* injection of different sources of manganese (Mn) on Divalent Metal Transporter 1 (*DMT1*) gene expression in the small intestine of broilers embryo and hatchling, and its subsequent effects on bone development. A total of 480 fertile eggs (ROSS 308) on embryonic day 17 were divided into 6 treatments with 3 replicates of 20 fertile eggs per replicate. Treatments were included: non-injected (NC) and diluent-injected controls (PC), manganese sulphate (MnS), manganese-methionine (Mn-Met), nano manganese (NMn) and nano manganese-methionine (NMn-Met) groups. Results showed that *in ovo* injection of different sources of Mn could change the *DMT1* mRNA level of intestinal cells (*p* < .05). *DMT1* mRNA level was up-regulated significantly (*p* < .05) by both nano forms of Mn on embryonic day 18 (E18) and post-hatch day 1 (P1) in all intestinal segments. On post-hatch day 7 (P7), bone length was higher in NMn treatment (*p* < .05) and tibia ash were affected by NMn and NMn-Met groups (*p* < .05). On (E18), the percentage of fresh and dry tibia weights were affected by Mn-Met, while on P14, the same characteristics were affected by NMn and MnS treatments, respectively (*p* < .05). The percentage of Mn in the tibiae ash content significantly (*p* < .05) enhanced by nano forms of Mn. It is concluded that *in ovo* injection of nano forms of Mn, either NMn or NMn-Met can be considered as a new strategy to improve Mn absorption in broilers intestine.

**HIGHLIGHTS**

- *In ovo* injection of nanoparticles led to positive effects on *DMT1* mRNA level in different intestinal segment of broilers embryo and hatching.
- *In ovo* injection of nanoparticles enhance the percentage of Mn in the tibiae ash content of broilers embryo and hatching.
- *In ovo* injection of Mn sources improved bone development.

**ARTICLE HISTORY**

Received 9 January 2019
Revised 15 July 2019
Accepted 16 July 2019

**KEYWORDS**

*DMT1* expression; embryo; *in ovo* injection; nano manganese-methionine; Tibia

**CONTACT** Dr. Majid Mottaghitalab m_mottaghi@gstp.ir Department of Animal Science, Faculty of Agriculture Science, University of Guilan, Rasht, Iran

© 2019 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**INTRODUCTION**

Manganese (Mn) is known as an essential and necessary trace element for enzyme activity, amino acid metabolism (Scott et al. 1976), growth, bone development and structure, perosis prevention, favourite quality of eggshell and performance in poultry (Olgun 2017). The consumption of Mn has become a main concern because of the rapid growth rate of commercial broiler strains, which puts additional stress on bone structure (Ji et al. 2006b). Keeping in mind that bone development is mainly programmed during embryogenesis and this leads to an additional requirement of chicken embryos for various nutrients, and subsequently the imbalance between reserves and requirement of nutrients stored within eggs may limit maximal growth and development of chicken embryos (Nordin et al. 1997).

It has been demonstrated that *in ovo* injection of nutrients could be considered as effective method to offer extra nutrients to embryo (Uni et al. 2005; Salary et al. 2014). Since nanoparticles of nutrient seem to be more efficient due to better delivery inside the tissues and cells, therefore *in ovo* injection may use as an option to ensure that nutrients reached to embryos. Yet, nanoparticles can bypass the physiological ways of nutrient transport across tissue and cell membranes, and protect compounds against destruction before reaching their targets (Sawosz et al. 2015).
Such findings led to the question of whether the in ovo injection of nanomanganese could promote availability and absorption of Mn in embryo, and help to bone formation either pre or post-hatch of broiler chicks.

Due to species-specific phenomenon of Manganese deficiency, and because birds absorb Mn, less efficiently than mammals do, poultry have relatively high requirements for Mn (Bai et al. 2008). The mechanisms of Mn absorption in different intestinal segments were not fully illustrated, and recently, researchers have focussed on the cellular mechanism involved in Mn transport. The divalent metal transporter 1 (DMT1) is a key protein in Mn uptake in the small intestine (Roth et al. 2002; Roth and Garrick 2003).

Mn release from the stomach into the duodenum is passed across the microvilli into enterocytes via DMT1 in mammals (Trinder et al. 2000; Knöpfel et al. 2005). DMT1 Overexpression in cultured cells results in an increase in Mn transport (Forbes and Gros 2003), and incubation with anti-DMT1 antibody stop up Mn absorption (Conrad et al. 2000). Those results are strongly supported by studies of the Belgrade (b) rat, which possesses a point mutation in DMT1 (G185R) that has a negative effect on Mn metabolism (Chua and Morgan 1997).

Bai et al. (2012) reported that manganese source affects its transport and gene expression of DMT1 in the broiler’s small intestine. For instance, prepared Mn from organic source showed higher DMT1 mRNA levels than MnSO₄ one. Consequently, compared to jejenum and ileum, DMT1 mRNA levels was much higher in the duodenum. Similarly, Liao et al. (2019) stated that broilers fed diets supplemented with Mn had higher duodenal DMT1 mRNA levels than those fed the control diet. Moreover, Mn chelates with moderate and strong chelation strength groups, appeared with higher duodenal DMT1 mRNA, than those weak chelation strengths and MnSO₄. These results support the idea that, DMT1, might be involved in the regulation of Mn transport in the proximal small intestine of broilers.

The aim of this study was to investigate the effects of in ovo injection of different sources of Mn on DMT1 expression in different intestinal segments and its subsequent effects on bone characteristics in broilers embryo and hatching.

Materials and methods

Material synthesis

Manganese sulphate (MnSO₄H₂O) (MnS) was purchased from MERCK, Germany. Green Nanotechnology Company (Guilan Science and Technology Park, Iran) provided manganese-methionine (Mn-Met), nano manganese (NMn) and nano manganese-methionine (NMn-Met).

Incubation

Total of 480 hatching eggs from a ROSS 308 commercial broiler breeder (Ramsar Toyoor Institute, Rasht, Iran) at age 61 weeks, were obtained with an average weight of 63.5 ± 1.25. After candling of all eggs, on embryonic day 17, the eggs were divided to six treatments with three replicates of 20 eggs per replicate. Treatments included: non-injected (NC) and 1 mL injection of solutions containing either saline as positive control (PC), or different preparation of manganese as: manganese sulphate (MnS), manganese- methionine (Mn-Met), 1 mL nano manganese (NMn), 1 mL nano manganese-methionine (NMn-Met) groups. All solutions for in ovo injection were prepared using different sources of Mn, so that to cover 10% of the Ross 308 recommendation for broiler chicks requirements. In such manner all in ovo supplemented Mn treatments, contain 12 mg/mL of Mn. The size of nano manganese and nano manganese-methionine particles were less than 100 nm (Based on SEM and TEM images). Either treatment solutions or sham control were injected into the amniotic fluid of the 17-day-old embryonic eggs (Keralapurath et al. 2010). On embryonic day 17, after second candling those eggs that were unfertilised or contained dead embryo, were omitted (Oliveira et al. 2015a). After injection, the experimental eggs placed to hatcher according to treatment replicate groups. The situation of the treatment replicated in the hatcher corresponded to their situation in the setter.

Birds and housing

After hatch, the day-old chicks housed at University of Guilan, Faculty of Agricultural Sciences, Education Research and Practice Farm, Rasht, Iran. Animal care and experimental procedures were approved by Iran veterinary organisation. The day-old chicks from the different treatment were allocated in 24 battery brooder cages. Each treatment had 8–10 birds depending on the hatch percentage of relevant replicate. Chicks were reared up to 2 weeks of age under a standard condition. Chicks fed corn-soybean meal-based diet to supply nutrient requirements of broilers; recommended by the Ross 308 broiler chicks’ catalogue. The manganese content in starter and grower
diets were 22.49 mg/kg and 20.34 mg/kg respectively. Feed and water were provided ad libitum.

Data collection

On embryonic day 18 (E18), 2 eggs from each replicate were randomly selected and their embryo were necropsied and gut and left tibia were removed. The duodenum, jejunum and ileum of the small intestine of each bird were used. The same sampling procedure was performed on post-hatch days 1 and 7 (P1 and P7), and the intestinal sections and left tibia from two birds randomly selected from each pen were removed. On post-hatch day 14 (P14), only tibia bones were sampled. The intestinal samples were quickly frozen in liquid nitrogen for assays of DMT1 gene expression. The left tibia of each chick was stripped of soft tissues and stored at -20°C until intended analyses.

Bones lengths were measured using a digital calliper (HB-102-111, Guanglu, China) (Oliveira et al. 2015b). All bones were first weighed in the presence of air and then dried at 100 °C for 24 h until no further weight loss was observed and weighed. Fresh and dry bone weights were calculated as percentages of body weight. Bone breaking strength was measured using a Lutron universal testing machine (FG5020, Hong Kong) at 50-kg load range with a crosshead speed of 50 mm/min as described by Oliveira et al. (2015b). After breaking strength measurement, the bones were ashed at 600 °C for 24 h, cooled in a desiccator, and weighed and tibia ash concentrations were calculated (Zhang and Coon 1997).

Mn concentrations in bone ash samples were measured using inductively coupled plasma spectroscopy (Youngli AAS 8020, Younglin, South Korea) as described by Lu et al. 2007). Approximately 0.2 g of each sample was weighed in triplicate and digested with 10 mL of HNO₃ and 0.4 mL of HClO₄ at 200 °C in a 50 mL calibrated flask until the solution cleared. Then, the solution was evaporated to approximately 0.2 mL, and diluted 1:10 with deionised water before analysis. Bovine liver powder as a standard reference material (Guilan Science and Technology Park, Rasht, Iran) was used for validation of the mineral analysis.

Table 1. Specific primer sequences for Real-time PCR.

| Gene | NCBI reference | Primer sequence | PCR product length | Annealing temperature |
|------|----------------|-----------------|-------------------|-----------------------|
| β-actin | NM_205518.1 | Forward 5'-GTCCAACCTCCTCCAGACATGT-3' Reverse 5'-ATAAGCCATGACCACTCG-3' | 169 | 63.6 |
| DMT1 | NM_001128102.1 | Forward 5'-CCATCCTCAGCGTCATCTACC-3' Reverse 5'-CACCTCCATCTCCACGGTC-3' | 191 | 61 |

DMT1 mRNA expression analysis

DMT1 levels in mucosal samples of different intestinal segments were analysed using quantitative real-time reverse transcription-PCR (Tchernitchko et al. 2002). RNA extraction procedure was carried out by TRizol reagent (Invitrogen, USA) based on the supplier’s instructions. The quality and quantity of extracted RNA was determined using NanoDrop- 2000 spectrophotometer (Thermo Scientific) and band’s quality of agarose gel electrophoresis, respectively. DNAse I treatment (Thermo Scientific) was applied to avoid possible contamination of genomic DNA. One microgram of the total RNA was reverse-transcribed to cDNA using Super Script III One-Step Reverse Transcript kit (Thermo Scientific). Specific primers of DMT1 and β-actin were designed using online software Primer 3 (Table 1). We used CFX96TM real-time detection system (Bio-Rad, USA) and 0.2 ml Thin-wall 8-tube strip with single attached optical flat caps (BIOpastics, Netherland, #B79201B) to obtain the optimum temperature of each gene primers and expression of target and internal control genes. Real-time PCR was carried out using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). Amplification was initiated by 180 s incubations at 94 °C, followed by 40 cycles at 94 °C for 45 s, annealing at the optimum temperature (depending on the optimised temperature of the each gene primers) for 40 s, and extension at 72 °C for 30 s. Data was normalised using Livak and Schmittgen (2001) formula (2-ΔΔCt).

Statistical analysis

Data analysis was done based on completely randomised design (CRD) using SAS software (SAS Institute 2011). Significance of means differences was tested using tukey multiple range test on a probability level of p < .05.

Results and discussion

DMT1 mRNA expression

The DMT1 mRNA expression results (Table 2) indicated that Mn metabolism in intestinal cells remarkably changed by Mn sources in different intestinal
segments. The *DMT1* mRNA level of Mn as nano sources were higher than that of Mn as organic and inorganic Mn. Moreover, ileum *DMT1* mRNA level was lower than duodenum and jejunum. On E18, the *DMT1* mRNA level in NMn-Met as nano organic source was significantly higher than organic and inorganic Mn in duodenum and jejunum, but, in ileum the expression of this gene was higher in another nano organic source (NMn) (*p* < .05). *DMT1* mRNA level in duodenum on P1 was higher in NMn treatment; however, in jejunum and ileum, NMn-Met treatment expressed higher gene expression (*p* < .05).

Compared to E18 and P1, *DMT1* mRNA level on P7 was decreased for all intestinal segments. *DMT1* gene expression in ileum was lower than two others intestinal segments. This result is in agreement with Bai et al. (2008) who found the *DMT1* mRNA levels in the duodenum and jejunum of broilers were higher than that in the ileum. This finding suggests that Mn absorption was a carrier-mediated process in the duodenum and jejunum, but was a non-saturable diffusion process in the ileum. Under the experimental conditions, results obtained by different mechanisms of Mn absorption in different intestinal regions suggest that the ileum is the major site of Mn absorption in the small intestine of broilers (Ji et al. 2006a; Bai et al. 2008).

*DMT1* mRNA level in the ileum in each period was lower than that in duodenum and jejunum, which may support those results that suggest Mn absorption is non-saturable diffusion process in the ileum. Thomson et al. (1971), who studied Mn absorption using whole-animal models, reported that diffusion was a main part of the absorptive process. In contrast, Testolin et al. (1993), using isolated perfused rat gut, observed that Mn absorption was a carrier-mediated saturable process. The same result was observed using in situ loops of rat intestine (Garcia-Aranda et al. 1983).

Two postulated factors that may affect Mn uptake from the broiler’s small intestine are: type and concentration of Mn in diets (Halpin et al. 1986). Based on reported data in a research (Bai et al. 2012), the sources of Mn should be viewed as a remarkable indicator in manganese transport and gene expression of *DMT1* in the small intestine of broilers. According to this, organic Mn sources revealed higher *DMT1* mRNA potential than MnSO4, and compared to jejunum and ileum, duodenum has the higher levels of *DMT1* mRNA. Similarly, the results of a recent study (Liao et al. 2019) confirmed that supplementation of broilers diets with Mn lead to higher duodenal *DMT1* mRNA levels than that control diet. Yet, Mn chelates with moderate and strong chelation strength groups, showed higher duodenal *DMT1* mRNA than either Mn chelates with weak chelation strength or MnSO4. Such results which are consistent with obtained data of present study, imply that, it is likely *DMT1* involve in Mn transport regulation in the proximal small intestine of broilers.

Ji et al. (2006a) using everted intestinal sacs found that Mn uptake was significantly affected by Mn sources, but no differences were found among various Mn sources in duodenal and jejunal sacs. However, the absorption of organic sources of Mn was greater than inorganic sources of Mn. Results from various studies revealed that organic Mn sources were more bioavailable than inorganic sources (Fly et al. 1989; Henry et al. 1989). In this study, broilers were fed a corn-soybean basal diet un-supplemented with Mn after hatch, therefore, Mn stores in the broilers’ body were depleted. However, Wedekind et al. (1991) fed broilers with a Mn-supplemented corn-soybean basal diet to examine the amount of Mn uptake in the small intestine. Due to Mn absorption might be affected by different factors such as fibre, phosphorus and some other minerals, some differences among experimental results may be occurred (Halpin et al. 1986).

Table 2. Effect of *in ovo* injection of different sources of manganese on *DMT1* mRNA Expression in different intestinal segments on embryonic day 18 and post-hatch days 1 and 7*.

| Treatments | E18 |  |  | P1 |  |  | P7 |  |
|------------|-----|-----|-----|-----|-----|-----|-----|-----|
|            | D   | J   | I   | D   | J   | I   | D   | J   | I   |
| NC         | 1.000<sup>a</sup> | 1.000<sup>a</sup> | 1.000<sup>a</sup> | 1.000<sup>b</sup> | 1.000<sup>b</sup> | 1.000<sup>b</sup> | 1.000<sup>c</sup> | 1.000<sup>c</sup> | 1.000<sup>c</sup> |
| PC         | 0.523<sup>b</sup> | 1.000<sup>b</sup> | 0.528<sup>d</sup> | 0.706<sup>d</sup> | 0.615<sup>d</sup> | 0.342<sup>b</sup> | 0.346<sup>d</sup> | 0.111<sup>c</sup> | 0.111<sup>c</sup> |
| MnS        | 1.180<sup>a</sup> | 2.209<sup>ab</sup> | 0.718<sup>b</sup> | 0.834<sup>d</sup> | 1.597<sup>bc</sup> | 0.403<sup>ab</sup> | 0.643<sup>d</sup> | 0.173<sup>c</sup> | 0.173<sup>c</sup> |
| Mn-Met     | 0.585<sup>b</sup> | 1.168<sup>b</sup> | 0.497<sup>d</sup> | 1.099<sup>bc</sup> | 2.112<sup>ab</sup> | 0.942<sup>ab</sup> | 1.051<sup>c</sup> | 0.508<sup>b</sup> | 0.508<sup>b</sup> |
| NMn        | 1.718<sup>b</sup> | 2.848<sup>a</sup> | 1.504<sup>a</sup> | 1.434<sup>a</sup> | 2.252<sup>ab</sup> | 0.842<sup>ab</sup> | 2.008<sup>a</sup> | 0.068<sup>c</sup> | 0.068<sup>c</sup> |
| NMn-Met    | 5.545<sup>a</sup> | 3.338<sup>a</sup> | 1.437<sup>a</sup> | 1.237<sup>ab</sup> | 2.962<sup>a</sup> | 1.217<sup>a</sup> | 1.432<sup>b</sup> | 0.178<sup>c</sup> | 0.178<sup>c</sup> |
| SEM        | 0.465 | 0.243 | 0.099 | 0.062 | 0.063 | 0.068 | 0.068 | 0.068 | 0.068 |
| p-value    | 0.003 | 0.000 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |

*Means within a column with different superscripts are significantly different (*p* < 0.05).

NC: Non-injected; PC: Diluent-injected controls; MnS: Manganese sulphate; Mn-Met: Manganese-methionine; NMn: nano manganese; NMn-Met: nano manganese–methionine; D: duodenum; J: jejunum; I: ileum; E18: embryonic day 18; P1: post-hatch days 1; P7: post-hatch days 7.
Bone characteristics

The results obtained from bone length, fresh and dry weight (Table 3) indicated that bone length was affected by treatment on P7 (p < 0.05). Fresh bone weight was affected by treatment on E18 and P14, and dried bone weight was affected by treatment on E18 and P7. Fresh and dry bone weights on E18 were higher in NMn group on P7 (p < 0.05). Also, fresh bone weight was affected by NMn group on P14 (p < 0.05).

Usind in ovo injection of Mn at different concentrations (20, 40 and 80 µg per egg) in quail hatching eggs, Yildiz et al. (2018), reported that compared to control (not injected), eggs treated with 40 and 80 µg of Mn, significantly increased the leg length, which is in agreement with that found on P7 of present experiment. In contrast, in ovo injection of mineral mixtures with different levels of Mn (from 0.013 to 0.039 mg per egg), did not affect tibia length in broilers (Oliveira et al. 2015b; Yair et al. 2015). Another published data by Yair et al. (2013) showed that in ovo injection of minerals, vitamins and carbohydrates did not significant effects on tibia length, fresh weight on E19, E21 and P3, though, in comparison to control, on P7 and P14, tibia appeared with higher length and more fresh weight was recorded on P14. These results are in agreement with those reported in this research.

Results from previous studies found that broiler breeders diets containing different levels of Mn (164 or 204 mg/kg) not impacted chick’s tibia length (Favero et al. 2013a, 2013b). Similarly, Bozkurt et al. (2015) reported that the inclusion of Mn at different levels (6.25, 12.50, 25 and 50 mg/kg) to broilers diets did not affect tibia length at the end of the trial. Discrepancy of data, may in part resulted due to the method of Mn administration, either in diet or in ovo injection.

The bone fresh and dry weight, which was calculated as a percentage of BW (Table 4), was also affected by treatments on E18 and P14 (p < 0.05). On E18, the percentage of bone fresh and dry weight of the birds from the Mn-Met group was higher than those of the other groups. On P14, the percentage of bone fresh weight of the birds from the NMn group and percentage of bone’s dry weight of the birds from the MnS group was higher than that of the other groups respectively. These results are in agreement with Yair et al. (2013) who observed significant effects on bone fresh weight on P14, however, reported data by other researches (Oliveira et al. 2015a, 2015b) are in contrast with above mentioned results.

### Table 3. Effect of injection of different sources of manganese on bone length (cm), fresh weight (gr) and dry weight (gr) on embryonic day 18 and post-hatch days 1, 7, 14

| Treatments | E18 | P1 | P7 | P14 |
|-----------|-----|----|----|-----|
|            | Length (cm) | Fresh (gr) | Dry (gr) | Length (cm) | Fresh (gr) | Dry (gr) | Length (cm) | Fresh (gr) | Dry (gr) | Length (cm) | Fresh (gr) | Dry (gr) |
| NC        | 2.345 | 0.209ab | 0.050ab | 3.104 | 0.388 | 0.130 | 4.431ab | 0.998 | 0.316ab | 5.110 | 3.007ab | 0.978 |
| PC        | 2.297 | 0.197b | 0.049b | 3.101 | 0.380 | 0.128 | 4.282b | 0.971 | 0.333b | 5.320 | 2.920b | 1.011 |
| MnS       | 2.325 | 0.203b | 0.049b | 3.116 | 0.390 | 0.130 | 4.111b | 0.983 | 0.336b | 5.115 | 3.203b | 1.000 |
| Mn-Met    | 2.397 | 0.208ab | 0.051ab | 3.116 | 0.408 | 0.133 | 3.354b | 1.033 | 0.308b | 5.314 | 3.124b | 1.072 |
| NMn       | 2.309 | 0.206ab | 0.055ab | 3.182 | 0.425 | 0.142 | 4.450b | 1.100 | 0.347b | 5.340 | 3.641b | 1.098 |
| NMn-Met   | 2.438 | 0.224* | 0.060* | 3.129 | 0.417 | 0.131 | 4.396b | 1.014 | 0.329ab | 5.203 | 3.055ab | 1.024 |
| SEM       | 0.022 | 0.003 | 0.0007 | 0.020 | 0.008 | 0.002 | 0.016 | 0.021 | 0.004 | 0.053 | 0.073 | 0.022 |
| p-value   | .192 | .033 | .016 | .888 | .469 | .959 | .025 | .536 | .044 | .696 | .042 | .520 |

*Means within a column with different superscripts are significantly different (p < 0.05).

NC: Non-injected; PC: Diluent-injected controls; MnS: Manganese sulphate; Mn-Met: Manganese-methionine; NMn: nano manganese; NMn-Met: nano manganese–methionine; E18: embryonic day 18; P1: post-hatch days 1; P7: post-hatch days 7; P14: post-hatch days 14.

### Table 4. Effect of injection of different sources of manganese on percentage of fresh and dry tibia weights and bone ash on embryonic day 18 and post-hatch days 1, 7, 14

| Treatments | E18 | P1 | P7 | P14 |
|-----------|-----|----|----|-----|
|            | Fresh (%) | Dry (%) | Ash (%) | Fresh (%) | Dry (%) | Ash (%) | Fresh (%) | Dry (%) | Ash (%) | Fresh (%) | Dry (%) | Ash (%) |
| NC        | 0.688* | 0.166ab | 0.209ab | 0.800 | 0.267 | 0.496 | 0.825 | 0.262 | 0.475a | 0.801ab | 0.260ab | 0.352 |
| PC        | 0.564* | 0.140b | 0.212b | 0.804 | 0.272 | 0.474 | 0.803 | 0.276 | 0.450ab | 0.847ab | 0.292ab | 0.387 |
| MnS       | 0.636b | 0.153b | 0.226b | 0.855 | 0.286 | 0.478 | 0.816 | 0.279 | 0.419b | 0.957ab | 0.328b | 0.386 |
| Mn-Met    | 0.710* | 0.174a | 0.210a | 0.815 | 0.268 | 0.497 | 0.865 | 0.258 | 0.371a | 0.883ab | 0.304ab | 0.313 |
| NMn       | 0.614b | 0.150b | 0.218b | 0.925 | 0.288 | 0.521 | 0.811 | 0.259 | 0.349ab | 0.966ab | 0.291ab | 0.340 |
| NMn-Met   | 0.671b | 0.162b | 0.232b | 0.852 | 0.267 | 0.481 | 0.819 | 0.268 | 0.372b | 0.860ab | 0.289ab | 0.361 |
| SEM       | 0.013 | 0.003 | 0.092 | 0.018 | 0.004 | 0.284 | 0.017 | 0.005 | 0.477 | 0.018 | 0.006 | 0.737 |
| p-value   | .011 | .015 | .940 | .360 | .546 | .998 | .872 | .652 | .025 | .034 | .048 | .878 |

*Means within a column with different superscripts are significantly different (p < 0.05).

NC: Non-injected; PC: Diluent-injected controls; MnS: Manganese sulphate; Mn-Met: Manganese-methionine; NMn: nano manganese; NMn-Met: nano manganese–methionine; E18: embryonic day 18; P1: post-hatch days 1; P7: post-hatch days 7; P14: post-hatch days 14.
During the embryonic period, the yolk (the main mineral source for the embryo) contains limited reserves of trace elements like Mn, and therefore the embryo consumes little of those minerals during that period (Yair and Uni 2011). Those minerals are important for broiler bone development (Angel 2007), so it is assumed that the prenatal mineral limitation will probably hinder bone development. The fastest development phase of the skeleton of the broiler occurs during the first two weeks of post-hatch age, and primarily during the first few days of age, when the bone is not completely formed. Micromineral consumption in the first few days of grow-out may be insufficient to cartilage ossification. In addition, a low mineral absorptive capacity of the broiler intestine during this period may exacerbate this insufficiency (Oliveira et al. 2015a). Therefore, it seems that in ovo injection of microminerals can improve the consumption and absorption of these substances in broiler embryo and improve bone development.

The percentage of ash in the bones of the birds belonging to the NMn and NMn-Met groups were significantly higher (p < .05) on P7 in comparison to the other treatments. In ovo injection of Mn sources did not exert different effects on bone breaking strength on other ages. Oliveira et al. (2015a) reported that by in ovo feeding of trace element, the percentage of bone ash of the birds belonging to the high mineral density group was higher than all other groups on day 1.

The bone breaking strength results (Table 5) exhibited that bone strength was not improved by the in ovo injection of diluent containing either supplemental Mn sources at any of the ages evaluated. It was expected that the higher ash content of the tibia would have been related with a higher bone breaking strength. However, the mechanical function of the tibia is not only determined by its composition, but also by its structure and confirmation (Sharir et al. 2008). These results are in agreement with those of Yair et al. (2013), who observed an increase in the ash content, but did not find a change in the mechanical properties of bones from 19 day of incubation through day 3 post-hatch. Bone mineralisation is not entirely complete at hatch; hence, although the mineral content of the bones may have increased, the bone may still not be entirely resistant to higher compression pressures. Manangi et al. (2012) reported that the supplementation of broiler chick diets with inorganic or organic Cu, Mn and Zn did not have different effects on bone breaking strength. These results were in agreement with Oliveira et al. (2015b) reported that in ovo injection of organic microminerals had no significant treatment effects on bone breaking strength.

The results obtained from Mn concentrations in the tibiae ash content (Table 6) indicated that percentage of Mn in the tibiae ash was improved by nano forms that may be due to higher bioavailability of this micro mineral. Moreover, Mn concentration in bone ash was in consistent with increasing the DMT1 activity in the intestine.

Oliveira et al. (2015a) observed the in ovo injection of minerals resulted in higher concentrations of Mn in the bone ash compared with control groups on day 1 and 7 post-hatch. Bello et al. (2014) reported that no significant changes were detected in bone Ca, P, Mg, or K when various levels of 25 (OH)D3 were administered by in ovo injection. In a previous study by Yair et al. (2013), it was found that injection of trace elements involved in bone development had positive effects on the concentration of Mn. Our results were in agreement with Yair and Uni (2011) and Yair et al. (2013), that reported that

### Table 5. Effect of injection of different sources of manganese on bone breaking strength (kg) on embryonic day 18 and post-hatch days 1, 7, 14.

| Treatments | E18 (kg) | P1 (kg) | P7 (kg) | P14 (kg) |
|------------|----------|---------|---------|----------|
| NC         | 0.401    | 0.944   | 2.872   | 5.752    |
| PC         | 0.403    | 0.913   | 2.886   | 5.658    |
| MnS        | 0.403    | 0.938   | 2.877   | 5.772    |
| Mn-Met     | 0.404    | 0.969   | 2.955   | 5.801    |
| NMn        | 0.405    | 0.954   | 2.957   | 5.845    |
| NMn-Met    | 0.409    | 0.996   | 2.941   | 5.772    |
| SEM        | 0.006    | 0.012   | 0.025   | 0.042    |
| p-value    | .910     | .236    | .827    | .801     |

*Means within a column with different superscripts are significantly different (p < .05).

NC: Non-injected; PC: Diluent-injected controls; MnS: Manganese sulphate; Mn-Met: Manganese-methionine; NMn: nano manganese; NMn-Met: nano manganese –methionine; E18: embryonic day 18; P1: post-hatch days 1; P7: post-hatch days 7; P14: post-hatch days 14.

### Table 6. Effect of injection of different sources of manganese on percentage of Mn in the tibiae ash on embryonic day 18 and post-hatch days 1, 7, 14.

| Treatments | E18 (%) | P1 (%) | P7 (%) | P14 (%) |
|------------|---------|--------|--------|---------|
| NC         | 0.009<sup>a</sup> | 0.005<sup>a</sup> | 0.005<sup>a</sup> | 0.005<sup>a</sup> |
| PC         | 0.008<sup>a</sup> | 0.005<sup>a</sup> | 0.005<sup>a</sup> | 0.005<sup>a</sup> |
| MnS        | 0.011<sup>b</sup> | 0.008<sup>b</sup> | 0.005<sup>b</sup> | 0.008<sup>b</sup> |
| Mn-Met     | 0.011<sup>b</sup> | 0.007<sup>b</sup> | 0.006<sup>b</sup> | 0.008<sup>b</sup> |
| NMn        | 0.012<sup>a</sup> | 0.009<sup>a</sup> | 0.010<sup>a</sup> | 0.010<sup>a</sup> |
| NMn-Met    | 0.012<sup>a</sup> | 0.009<sup>a</sup> | 0.007<sup>a</sup> | 0.009<sup>a</sup> |
| SEM        | 0.0002   | 0.0002  | 0.0002  | 0.0002  |
| p-value    | .001     | .001    | .001    | .001    |

*Means within a column with different superscripts are significantly different (p < .05).

NC: Non-injected; PC: Diluent-injected controls; MnS: Manganese sulphate; Mn-Met: Manganese-methionine; NMn: nano manganese; NMn-Met: nano manganese –methionine; E18: embryonic day 18; P1: post-hatch days 1; P7: post-hatch days 7; P14: post-hatch days 14.
bone development and their subsequent properties in broilers are affected by nutrient availability during the embryonic and post-hatch periods. Bao et al. (2007) fed broilers with different sources and levels of organic Cu, Fe, Mn and Zn, and reported that there were no differences in the concentration of these minerals in the bones of birds that received either inorganic minerals or high concentrations of organic minerals. In order to achieve appropriate bone mineralisation during the embryonic phase, the concentration of the minerals used as a substrate for ossification by osteocytes must be at sufficient levels. Yair and Uni (2011) reported that bone concentrations of Ca and P are not reduced as are the concentrations of Zn, Cu and Mn between 17 and 21 day of incubation. Reduced concentrations of these microminerals may restrict the ossification process of cartilage during the last days of incubation and during the first few days post-hatch. Improvements in the concentrations and sources of available microminerals (i.e. Zn, Cu and Mn) may be associated with an increase in tibia ash, particularly as these microminerals are used as building blocks of metalloenzymes necessary for connective tissue synthesis. Based on our results, the injection of Mn enriched solutions has the potential to improve Mn absorption in broilers’ intestine and bone mineralisation. Further research to determine effects of in ovo injection of Mn sources and levels on post-hatch performance, bone development, and bone mineralisation in broilers should be considered.

Conclusions
The expression profile of DMT1 were evaluated in different intestinal segments of broilers. We found that different sources of Mn can change Mn absorption using increase DMT1 mRNA level as a carrier-mediated process in broilers intestine. This may be due to the higher bioavailability of this sources of mineral. The Percentage of Mn in the tibiae ash indicated that percentage of Mn was affected by nano sources of Mn in all periods. The results of this study showed that in ovo injection of Mn nanoparticle has the potential to improve bone mineralisation and bone development during embryonic phase and first few days post-hatch.

Disclosure statement
No potential conflict of interest was reported by the authors.

Funding
Authors are grateful to Ramsar Toyoor Institute for the financial support.

References
Angel R. 2007. Metabolic disorders: limitations to growth of and mineral deposition into the broiler skeleton after hatch and potential implications for leg problems. J Appl Poult Res. 16:138–149.
Bai SP, Lu L, Luo XG, Liu B. 2008. Kinetics of manganese absorption in ligated small intestinal segments of broilers. Poult Sci. 87:2596–2604.
Bai SP, Lu L, Wang RL, Xi L, Zhang LY, Luo XG. 2012. Manganese source affects manganese transport and gene expression of divalent metal transporter 1 in the small intestine of broilers. Br J Nutr. 108:267–276.
Bao YM, Choct M, Jii PA, Bruerton K. 2007. Effect of organically complexed copper, iron, manganese, and zinc on broiler performance, mineral excretion, and accumulation in tissues. J Appl Poult Res. 16:448–455.
Bello A, Bricka RM, Gerard PD, Peebles ED. 2014. Effects of commercial in ovo injection of 25-hydroxycholecalciferol on broiler bone development and mineralization on days 0 and 21 posthatch. Poult Sci. 93:1053–1058.
Bozkurt Z, Bulbul T, Bozkurt MF, Bulbul A, Maralcan G, Queklooglu K. 2015. 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. Kafkas Univ Vet Fak Derg. 21:623–630.
Chua AC, Morgan EH. 1997. Manganese metabolism is impaired in the Belgrade laboratory rat. J Comp Physiol B, Biochem Syst Environ Physiol. 167:361–369.
Conrad ME, Umbreit JN, Moore EG, Hainsworth LN, Porubcin M, Simovich MJ, Nakada MT, Dolan K, Garrick MD. 2000. Separate pathways for cellular uptake of ferric and ferrous iron. Am J Physiol Gastrointest Liver Physiol. 279:G767–774.
Favero A, Vieira SL, Angel CR, Bess F, Cemim HS, Ward TL. 2013a. Reproductive performance of Cobb 500 breeder hens fed diets supplemented with zinc, manganese, and copper from inorganic and amino acid-complexed sources. J Appl Poult Res. 22:80–91.
Favero A, Vieira SL, Angel CR, Bos-Mikich A, Lothhammer N, Taschetto D, Cruz RF, Ward TL. 2013b. Development of bone in chick embryos from Cobb 500 breeder hens fed diets supplemented with zinc, manganese, and copper from inorganic and amino acid-complexed sources. Poult Sci. 92:402–411.
Fly AD, Izquierdo OA, Lowny KR, Baker DH. 1989. Manganese bioavailability in a Mn-methionine chelate. Nutr Res. 9:901–910.
Forbes JR, Gros P. 2003. Iron, manganese, and cobalt transport by Nramp1 (Slc11a1) and Nramp2 (Slc11a2) expressed at the plasma membrane. Blood. 102: 1884–1892.

Ethical approval
Animal care and experimental procedures were under the regulation of Iranian veterinary organization and approved by University of Guilan.
Garcia-Aranda JA, Wapnir RA, Lifshitz F. 1983. *In vivo* intestinal absorption of manganese in the rat. J Nutr. 113: 2601–2607.

Halpin KM, Chausow DG, Baker DH. 1986. Efficiency of manganese absorption in chicks fed corn-soy and casein diets. J Nutr. 116:1747–1751.

Henry PR, Ammerman CB, Miles RD. 1989. Relative bioavailability of manganese in a manganese-methionine complex for broiler chicks. Poult Sci. 68:107–112.

Ji F, Luo XG, Lu L, Liu B, Yu SX. 2006a. Effects of manganese source and calcium on manganese uptake by in vitro everted gut sacs of broilers' intestinal segments. Poult Sci. 85:1217–1225.

Ji F, Luo XG, Lu L, Liu B, Yu SX. 2006b. Effect of manganese source on manganese absorption by the intestine of broilers. Poult Sci. 85:1947–1952.

Keralapurath MM, Keirs RW, Corzo A, Bennett LW, Pulikanti R, Peebles ED. 2010. Effects of *in ovo* injection of L-carnitine on subsequent broiler chick tissue nutrient profiles. Poult Sci. 89:335–341.

Knöpfel M, Zhao L, Garrick MD. 2005. Transport of divalent transition-metal ions is lost in small-intestinal tissue of b/b Belgrade rats. Biochemistry. 44:3454–3465.

Liao XD, Wang G, Lu L, Zhang LY, Lan YX, Li SF, Luo XG. 2019. Effect of manganese source on manganese absorption and expression of related transporters in the small intestine of broilers. Poult Sci. pii: pe2293.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25:402–408.

Lu L, Luo XG, Ji C, Liu B, Yu SX. 2007. Effect of manganese supplementation and source on carcase traits, meat quality, and lipid oxidation in broilers. J Anim Sci. 85:812–822.

Luo XG, Li SF, Lu L, Liu B, Kuang X, Shao GZ, Yu SX. 2007. Gene expression of manganese-containing superoxide dismutase as a biomarker of manganese bioavailability for manganese sources in broilers. Poult Sci. 86:888–894.

Manangi MK, Vazquez-Anon M, Richards JD, Carter S, Buresh RE, Christensen KD. 2012. Impact of feeding lower levels of chelated trace minerals versus industry levels of inorganic trace minerals on broiler performance, yield, footpad health, and litter mineral concentration. J Appl Poult Res. 21:881–890.

Nordin BE, Gurr MI, McIntosh GH, Schaafisma G, Miller GD, Groziak SM, Sieber R, Goulding A. 1997. Dietary calcium in health. Bull Int Dairy Fed. 322:36–40.

Olgun O. 2017. Manganese in poultry nutrition and its effect on performance and eggshell quality. Worlds Poult Sci J. 73:45–56.

Oliveira TF, Bertechini AG, Bricka RM, Hester PY, Kim EJ, Gerard PD, Peebles ED. 2015a. Effects of *in ovo* injection of organic trace minerals and post-hatch holding time on broiler performance and bone characteristics. Poult Sci. 94:2677–2685.

Oliveira TF, Bertechini AG, Bricka RM, Kim EJ, Gerard PD, Peebles ED. 2015b. Effects of *in ovo* injection of organic zinc, manganese, and copper on the hatchability and bone parameters of broiler hatchings. Poult Sci. 94: 2488–2494.

Roth JA, Garrick MD. 2003. Iron interactions and other biological reactions mediating the physiological and toxic actions of manganese. Biochem Pharmacol. 66:1–13.

Roth JA, Horbicki C, Higgins D, Lein P, Garrick MD. 2002. Mechanisms of manganese-induced rat pheochromocytoma (PC12) cell death and cell differentiation. Neurotoxicology. 23:147–157.

Salay J, Sahebi-Ala F, Kalantar M, Matin HR. 2014. *In ovo* injection of vitamin E on post-hatch immunological parameters and broiler chicken performance. Asian Pac J Trop Biomed. 4:616–619.

SAS Institute. 2011. SAS/IML 9.3 user's guide. Cary, NC: SAS Institute.

Sawosz F, Pineda L, Hotowy A, Hyttel P, Sawosz E, Szmidt M, Niemiec T, Chwalibog A. 2012. Nano-nutrition of chicken embryos. The effect of silver nanoparticles and glutamine on molecular responses, and the morphology of pectoral muscle. Baltic J Comp Clin Syst Biol. 2:29–45.

Scott ML, Nesheim MC, Young RJ. 1976. Nutrition of the chicken. New York (NY), Ithaca: ML Scott and Associates.

Sharir A, Barak MM, Shahar R. 2008. Whole bone mechanics and mechanical testing. Vet J. 177:8–17.

Tchemitchko D, Bourgeois M, Martin ME, Beaumont C. 2002. Expression of the two mRNA isoforms of the iron transporter Nramp2/DMT1 in mice and function of the iron responsive element. Biochem J. 363:449–455.

Testolin G, Ciappellano S, Alberio A, Piccinini F, Paracchini L, Jotti A. 1993. Intestinal absorption of manganese: an in vitro study. Ann Nutr Metab. 37:289–294.

Thomson AH, Olutunbosun D, Valverg LS. 1971. Interrelation of intestinal transport system for manganese and iron. J Lab Clin Med. 78:642–655.

Trinder D, Oates PS, Thomas C, Sadleir J, Morgan EH. 2000. Localisation of divalent metal transporter 1 (DMT1) to the microvillus membrane of rat duodenal enterocytes in iron deficiency, but to hepatocytes in iron overload. Gut. 46: 270–276.

Uni Z, Ferket PR, Tako E, Kedar O. 2005. *In ovo* feeding improves energy status of late-term chicken embryos. Poult Sci. 84:764–770.

Wedekind KJ, Titgemeyer EC, Twardock AR, Baker DH. 1991. Phosphorus, but not calcium, affects manganese absorption and turnover in chicks. J Nutr. 121:1776–1786.

Yair R, Shahar R, Uni Z. 2013. Prenatal nutritional manipulation by *in ovo* enrichment influences bone structure, composition, and mechanical properties. J Anim Sci. 91: 2784–2793.

Yair R, Shahar R, Uni Z. 2015. *In ovo* feeding with minerals and vitamin D3 improves bone properties in hatchlings and mature broilers. Poult Sci. 94:2695–2707.

Yair R, Uni Z. 2011. Content and uptake of minerals in the yolk of broiler embryos during incubation and effect of nutrient enrichment. Poult Sci. 90:1523–1531.

Yildiz AO, Şentürk ET, Aşgün A, Olgun O. 2018. Effects of *in ovo* injection of manganese on some organ weights and lengths in quail hatching eggs. Selcuk J Agr Food Sci. 32: 345–349.

Zhang BI, Coon CN. 1997. The relationship of various tibia bone measurements in hens. Poult Sci. 76:1698–1701.

Zielinska M, Sawosz E, Grodzik M, Balcerak M, Wierzbicki M, Skomial J, Sawosz F, Chwalibog A. 2012. Effect of taurine and gold nanoparticles on the morphological and molecular characteristics of muscle development during chicken embryogenesis. Arch Anim Nutr. 66:1–13.