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Effects of *in ovo* Injection of Zinc Acetate on some Gene Expression Associated with Embryonic Growth and Development, and with Growth and Carcass Characteristics of the Resultant Chicks

Research Article

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ABSTRACT

This study was conducted in two steps to determine the effects of *in ovo* injection of zinc acetate (ZAC) on some gene expression associated with embryonic growth and development, and with growth and carcass characteristics of the resultant chicks. In the first step the effect of *in ovo* injection of ZAC on the expression of insulin-like growth factors (IGFs:IGF-I and IGF-I), myogenic regulatory factors (MRFs) and metallothionein (MT) genes was examined, and in the step two, the associations between genes expression with fertility, embryonic mortality, hatchability, body weight (BW) and relative organ weight on day of hatch, and body weight (BW) and feed conversion ratio (FCR) a week after hatching were investigated. Seven hundred twenty fertile eggs (n=720) were randomly divided into 4 treatment groups consisted of: 1) non-injected (NI) group, 2) sham group (group injected with deionized water (DW)), and 3) and 4) groups injected with 3.64 and 5.23 M ZAC, respectively. On day 3 of incubation the eggs were injected with 50 µL of each treatment into the albumen. On day 5 of the incubation and on hatch day, liver and breast muscle of the chicks were removed and immediately stored at -80 ˚C until RNA extraction and real time-PCR analysis. *In ovo* ZAC injection increased myogenic differentiation factor both in embryo and breast muscle. MT mRNA expression was also increased by ZAC injection. Hatchability was lower in ZAC groups and early embryonic mortality was higher in sham control (SHC) and ZAC groups. Injection of ZAC improved both BW and FCR during the first week of rearing, and increased the relative weights of liver and breast muscle. In conclusion, *in ovo* injection of ZAC increased some of gene expressions of tissues and BW7 during the first week, but decreased hatchability. Hence, lower doses of ZAC are recommended to use in further experiments.

KEY WORDS chickens, growth and development, *in ovo* injection, insulin-like growth factors, myogenic regulatory factors, zinc.

INTRODUCTION

Skeletal muscle forms the largest proportion of animal mass. So one of the aims of the poultry industry has been to select animals to increase growth and muscle mass (Velleman, 2007). Muscle growth is part of the process of growth. Muscle growth during the embryonic period of growth and development occurs through hyperplasia and hypertrophy (Smith, 1963; Musawi *et al*. 2011). Hyperplasia increased myoblast cell number during the embryonic period (Smith, 1963; Velleman, 2007). After proliferating, cells begin to withdraw from the cell cycle and form multinucleated myotube (Olson, 1992; Musawi *et al*. 2011) and muscle fibres (Smith, 1963; Musawi *et al*. 2011). Growth factor and myogenic regulatory factors are strong stimulators of myoblast cell proliferation and differentiation

(Velleman, 2007). The MRFs are family of basic Helix-Loop-Helix (bHLH) transcription factors that include MYOD, MYF-5, myogenin, and myogenic regulatory factor- 4 (MRF-4) (Kielbowna and Jedrzejowska, 2012). MYOD and MYF-5 are the first factors expressed during myoblast proliferation, myogenin and MRF-4 are second factors expressed during myotube formation and differentiation (Richter *et al*. 2002). MYOD and MYF-5 both play a role in the specification, formation and maintenance of myoblasts (Pownall *et al*. 2002; Richter *et al*. 2002; Parker *et al*. 2003).

In chicken embryo, skeletal muscle development beings at 2.5 embryonic days. On 6th and 7th day of embryonic stage, myoblasts begin to exit from the cell cycle and primary myotube formation beings. During 10-11th days of embryonic development a second wave of myoblasts stop proliferation, migrate, and fuse into myofibers determined the secondary myogenic period in chicken embryos (Christ, 1977; O'Neill, 1987). MYOD and MYF-5 express in chicken embryo almost at the same time and then myogenin and MRF-4 expresses. Richter *et al*. (2002) have observed MYOD, MYF-5 and MRF-4 in chicken embryo expression on 2nd day of embryonic development and myogenin expression after these genes on the 3^{d} day. Among the growth factors, insulin-like growth factors (IGF-I and IGF-II) enhance proliferation and differentiation of myoblasts through the regulation of MRFs expression and activated insulin-like growth factor receptor-1 (IGF-1R) (Engert *et al*. 1996; Coolican *et al*. 1997). IGF-I and IGF-II gene for the first time expressed during the blastoderm stage of chicken embryo development. Then IGF-I mRNA levels gradually reduced and on first of embryonic stage remained low until the 10th day. Levels then increased on the 11th day of embryonic and remained high until 13th days of embryonic. IGF-I mRNA reduced in 14th day of embryonic and remained at this level until the 18th day. Levels reduced prior to hatching. A mount IGF-II mRNA fluctuated from day 0 of embryonic development to 20th day. Levels between day 0 and 5th days of embryonic stage were high and then reduced to 16th day (Kocamis and Killefer, 2003). After hatching, IGF-I levels increased and IGF-II levels decreased (Lu *et al*. 2007). IGF-I induces myoblast proliferation and stimulates myogenic differentiation (Engert *et al*. 1996; Quinn *et al*. 1994). IGF-II is produced by myoblast cells as they differentiate. The rate and amount of IGF-II expression correlated with the differentiation rate (Florini *et al*. 1991).

Injection of recombinant human IGF-I (rhIGF-I) on 3 days of incubation improved growth, tissue development and feed efficiencies in the broiler (Kocamis *et al*. 1998) because IGF-I affected the expression of MRFs (Richter *et al*. 2002). So part of embryonic muscle growth mediated by IGFs and MRFs genes expression, separately or by IGF-I effect on MRFs. Growth and development of embryo are dependent on the nutrient and nutritional inadequacy reduces growth.

The dietary nutrients have most effect on the growth, development and viability of the avian embryo (Wilsons, 1997). The inadequate, unbalanced or excessive levels of nutrients in egg cause the death of the embryo (Wilsons, 1997). One of these nutrients is Zn diets that are deficient in Zn can lead to a decrease in fertility, hatchability, egg production and increase early and late embryonic mortality (Kienholz *et al*. 1961; Hudson *et al*. 2004). In contrast, supplementation of Zn in diets, increase fertility, egg production, hatchability and reduced early and late embryonic mortality (Hudson *et al*. 2004; Stanley *et al*. 2012; Favero *et al*. 2013). Stanley *et al*. (2012) observed no significant effect on hatchability and Savage (1968) observed Zn decreased hatchability and increased early embryonic mortality because Zn affected copper deficiency then increased anemia and a high incidence of hemorrhage. Joshua et al. (2016) observed, *in ovo* feeding of nano minerals were not harmful to the developing embryo and did not influence the hatchability and feed efficiency were best for nano forms of zinc, copper and selenium. In Hudson *et al*. (2004) investigation, adding of Zn in diet of broiler breeder hens, had no influence on the chick weight (BW) and organ weight (liver, pipping muscle and etc.).

Dietary supplementation of Zn affected the body weight gain (BWG), feed intake, feed conversion ratio, immunity response and improved performance in broilers (Kucuk *et al*. 2003; Sahin *et al*. 2006; Norouzi *et al*. 2013; Ezzati *et al*. 2013). Although, accumulation of Zn and Mn in hepatic tissue are related to their levels of supplementation in diets (Shyam Sunder *et al*. 2013) but some studies demonstrated that Zn in broiler diet did not effected BW and feed conversion ratio (Saenmahayak *et al*. 2012; Shyam Sunder *et al*. 2013; Ezzati *et al*. 2013).

Zn deficiency reduced growth hormone (GH), growth hormone receptor (GHR), IGF-I mRNA expression in animal and human (MacDonald, 2000; Yu *et al*. 2005; Imamoglu *et al*. 2005; Hamza *et al*. 2012). Supplementation with Zn increased GH, GHR, IGF-I, growth and feed intake (MacDonald, 2000; Yu *et al*. 2005), but in some studies did not significantly change the body weight (Imamoglu *et al*. 2005; Hamza *et al*. 2012). Wang *et al*. (2002) observed low Zn in newly hatched chicken reduced IGF-I and supplementation of Zn in diet did not induce any difference between Zn deficient and Zn adequate birds.

In ovo injection of Zn on the 14th days of incubation into the yolk sac/amnion of the chicken embryos affected IGF-I and IGF-II expression in liver. Zn did not show any difference in the expression of IGF-I on the 18th day of incuba-

tion and day of hatch but the expression was decreased on the 20th day of incubation. The expression of IGF-II was significantly increased on the 18th and 20th day of incubation but the expression was decreased on day of hatch (Akshat *et al*. 2012). Paulk *et al*. (2014) showed increased Zn in the diet of pigs, reduced IGF-I and IGF-1R expression and there was no effect on the expression of MYOD, MYF-5 and muscle fiber cross-sectional area.

Metallothioneins (MT) are proteins which bind heavy metals like Zn with high affinity (Fernando *et al*. 1989). In some studies of the avian MT, a single isoform of MT (Sandrock *et al*. 1983; Mccormick, 1984; Fernando *et al*. 1989; Dong *et al*. 2008) and this isoform was found in liver, pancreas, kidney, mucosal (Oh *et al*. 1979; Mccormick, 1984; Cao *et al*. 2002; Shen *et al*. 2013) and in embryo (Sandrock *et al*. 1983). Hepatic and mucosal MT increased with supplemented Zn and age (Cao *et al*. 2002).

Levels of hepatic MT mRNA induced by Zn, Cd and Cu (Fernando *et al*. 1989; Andrews and Fernando, 1991; Coa *et al*. 2002) and chicken liver produced apparently a single form of metallothionein upon heavy metal exposure (Chakraborty *et al*. 1987). MT mRNA was low in embryonic liver and increased during first week after hatching. Before decreasing again to the basal levels found in adult liver (Sandrock *et al*. 1983; Fernando *et al*. 1989).

Fernando *et al*. (1989) study showed that *in ovo* injection of 2.4 and 4.8 mg metal combination of $ZnCl_2$ and $CuCl_2$ on the 13th and 18th day of incubation in to the air sac, increased MT mRNA expression 8 hours after injection and after hatch. But 4.8 mg metal composition was toxic and 80% embryos died after 10 hours.

Fleet and Mccormick (1988) injected Zn in eggs prior to incubation (0.2, 0.4 and 0.6) and on the 17th day of incubation (0.55, 2.75 and 4.95) in yolk sac and observed increasing MT levels in chick liver.

The aim of this study was to examined the effect of zinc acetate *in ovo* injection in early embryonic stage on growth and development of embryonic muscle by measuring IGF-I, IGF-II, IGF-1R, MYOD and MYF-5 mRNA relative expression, chick characteristics (BW and relative organ weight), BW7 and FCR in seven days after hatch and responsiveness of hepatic MT levels in liver at hatch to supplementation of different amount of ZAC.

MATERIALS AND METHODS

The experiment was approved by the Ethical Commission for Experimental Use of Animals of Sari Agricultural Sciences and Natural Resources University.

Seven hundred and twenty eggs were used in a completely randomized design with 4 treatments, 5 replicates and 36 eggs in each replicate at Zarpa Company. Fertile eggs were numbered, weighted and incubated at 37.5 ˚C (85.5% relative humidity).

Solution

The solution was prepared under biological hood. 1.6 and 2.3 g Zn acetate (Merck Company) were dissolved in 50 Ml deionize water (respectively 3.64 and 5.23 M). This solution and deionize water were autoclaved for 20 min and 120 ˚C.

Treatment

Fertile eggs were randomly assigned to 4 treatments including, non- injected control (NIC), sham control (SHC), injected 3.64 and 5.23 M Zn acetate (respectively ZAC 3.64 and ZAC 5.23) then numbered and weighed and incubated at 37.5˚C (85.5% relative humidity). On 3th day of embryonic incubation DW, ZAC 3.64 and ZAC 5.23 were injected at 50 µL into albumen. Prior to injection, the injection site or narrow end of the egg was sterilized with ethyl alcohol (70% ethanol) and this site was punched with 21-gauge needle. Then solution and DW was injected with 22-gauge needle. The hole was sealed with a paraffin sticker and the eggs were placed in incubator.

Tissue sampling

On 5th day of embryonic incubation, 2 eggs from each replicate (ten eggs from each treatment) were collected and all embryos washed free yolk, albumin and other embryonic membranes by DW. All embryos were placed in tubes. The samples were quickly frozen in liquid nitrogen. Then were transferred and stored at -80 ˚C until used for determination of relative genes expression. After 21 days, newly hatched chicks were weighed and two chicks from each replicate (ten chicks from each treatment) were killed by cervical dislocation. Then samples of liver and breast muscle were removed and stored at -80 ˚C for evaluation of relative genes expression.

RNA extraction

Total RNA was extracted from the embryo, liver and breast according to the accuZol™ user's manual (Bioneer Co). AccuZol™ is a ready-to-use reagent for the isolation of total RNA from tissues. Before adding AccuZol™, tissues were homogenized with liquid nitrogen and then mixed with this reagent. Samples of RNA were stored at -80 ˚C. Before stored, RNA integrity was electrophoretically verified using ethidium bromide. Then DNase (DNaseI, RNase-free for CinnaGen Co) was used to remove DNA.

Reverse transcription

RNA was converted to cDNA by cDNA synthesis kit (AccuPower® CycleScript RT PreMix (dN_6) (Bioneer Co)).

This product contains all components including thermostable cyclescript reverse transcriptase, dNTPs, reaction buffer, primer and stabilizers for reveres transcription.

According to the manufacture's instruction, only RNA and nuclease-free water were added to this product.

Primers

All PCR primers were obtained from SinaClon (or CinnaGen Co). Primers for MYOD, MYF-5 and MT were designed on the basis of published sequences for chick for Vector NTI Advance™ Software (Table 1). Then primers were diluted by adding distilled water. PCR and real-time PCR products size were confirmed by agarose gel electrophoresis and ethidium bromide staining.

Real-time PCR

For the real-time PCR reaction, 1 µL of cDNA was added to 14 µL of solution containing 2 µL forwards and reverse primer, 7.5 µL Green PCR Master Mix (QuantiNova^{TM} SYBR Green PCR kit) and 4.5 µL RNase-free water. Then solutions were prepared, with any IGFs, MRFs and MT genes, one ß-actin (housekeeping) sample was placed in the Rotor-Gene 3000 (Corbett Company). Forty cycles of amplification were performed. Each cycle consisted of predenaturation at 95 ˚C for 5 min followed by denaturation at 95 ˚C for 10 min and annealing and extension at 60 ˚C for 30 s. The comparative Ct value method was employed to determine expression concentrations for target genes relative to those for β-Actin gene. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression determined from real-time qualitative PCR experiments as described by Livak and Schmittgen (2001).

Fertility, hatchability, mortality

After 21 days, the number of chick was determined to calculate the hatchability of eggs set (except for 5th day) and fertile eggs. Then unhatched eggs were broken open to determine unfertile eggs to assess the fertility and to determine the age at death (Hamburger and Hamilton, 1951; Cobb, 2001) , presented as early (1 to 7 d), mid (8 to 14 d), or late (15 to 21) embryonic mortality.

Hatchability of eggs set %= (number of chicks hatched/number of eggs set) × 100

Hatchability of fertile %= (number of chicks hatched/number of fertile eggs set) × 100

Fertility %= (number of fertile eggs/number of total egg set) × 100.

Body weight, relative organ weight and feed conversion ratio

Newly hatched chicks were weighed and 2 chicks from each replicate (ten chicks from each treatment) were killed by cervical dislocation. Liver, breast and thigh were collected immediately after the chicks were killed and weighed. Relative organ weight were calculated as a percentage of body weight (relative organ weight=(organ weight (g)/body weight (g))×100).

Remaining chicks received leg number and were transferred in farm of agriculture and Natural Resources University of Sari (Iran). Each treatment was randomly divided to 4 replicates. All birds were fed basal diet (starter diet) and free access to water for the first week (Table 2). Feed intake was measured during experiment and mortality was recorded daily. After the first week chicks were weighed and feed: gain was measured.

Statistical analysis

Data were analysed according to a completely randomized design using the Proc GLM (SAS, 2002). Before analysis data were normalized by JMP 7. Duncan's multiple range tests were conducted to compare the effect among different treatments ($P<0.05$).

RESULTS AND DISCUSSION

The results are summarised in Tables 3 to 7.

IGFs and MRFs relative expression in embryo tissue

In the present study, *in ovo* injection of ZAC was increased significantly expression IGF-1 and MYOD ($P<0.05$). IGF-2 and MYF-5 mRNA expression was not ($P>0.05$) affected by ZAC treatment.

However, the expression levels of IGF-R were significantly decreased ($P<0.05$) by ZAC *in ovo* injection treatment compared to non-injected control and were not significantly ($P>0.05$) affected by ZAC *in ovo* injection treatment compared to sham control (Table 3).

IGFs and MRFs relative expression in muscle (breast) tissue

The MYOD and MYF-5 mRNA expression was increased respectively, 3.64 M and 5.23 M zinc acetate *in ovo* injection ($P<0.05$). IGF-2 mRNA and IGF-R mRNA expression in muscle was not affected by ZAC ($P>0.05$). However, IGF-1 mRNA expression in this tissue decreased by ZAC *in ovo* injection treatment compared to non-injected control ($P<0.05$) and was not significantly affected by ZAC *in ovo* injection treatment compared to sham control ($P>0.05$; Table 4).

Metallothionein relative expression in liver

Metallothionein mRNA expression was increased by 3.64 and 5.23 M zinc acetate *in ovo* injection ($P<0.05$). So that by increasing the amount of Zn injection, gene expression was increased (Table 5).

Table 1 Primer sequences used for real-time PCR

Primer	Direction	Sequence (5′-3′)	Size	Accession no.
IGF-1	Forward	TGTACTGTGCTCCAATAAAGC	127	NM_001004384.2
	Reverse	CTGTTTCCTGTGTTCCCTCTACTTG		
IGF-2	Forward	TGTGGAGGAGTGCTGCTTTC	101	NM_001030342.1
	Reverse	GGGAGGTGGCGGAGAGGTCA		
IGF-R	Forward	CTCTTCCCCAACCTCACGGTCA	274	S40818
	Reverse	GCTTCTCCTCCATCGTTCCTGG		
MYOD	Forward	GCTACTACACGGAATCACCA	198	NM_204214.1
	Reverse	GGGCTCCACTGTCACTCA		
MYF-5	Forward	GAGGAGGAGGCTGAAGAAAG	174	NM_001030363.1
	Reverse	CCCGGCAGGTGATAGTAGTT		
MT	Forward	TGCTGCTGGTGACTCCTGCT	139	NM_205275.1
	Reverse	CggTTCCTTgCAgACACAgc		

Table 2 Composition of basal starter diet

Item	1 to 7 days
Crude protein (%)	21- 21.5
Ca (%)	1.01
Available P (%)	0.49
Energy (KJ/kg)	11925.5

Table 3 The effect of *in ovo* injection of zinc acetate (ZAC) on insulin-like growth factors (IGFs) and myogenic regulatory factors (MRFs) mRNA expression in embryo

Variable	Treatments				SEM	P-value
	NIC	SHC	ZAC 3.64	ZAC 5.23		
IGF-I	1.00±0.02^{b}	1.45±0.24^{b}	1.99±0.79^{b}	5.71±0.9^{a}	0.304	0.0005
IGF-II	1.2±0.34^{a}	3.8±2.00^{a}	6.45±3.60^{a}	12.95±4.44^{a}	1.516	0.087
IGF-R	1.07±0.22^{a}	0.05±0.02^{b}	0.25±0.1^{b}	0.1±0.03^{b}	0.060	0.294
MYOD	1.05±0.19^{b}	1.52±0.27^{b}	2.87±0.54^{a}	0.66±0.2^{b}	0.166	0.0028
MYF-5	1.08±0.21^{a}	1.48±0.13^{a}	12.82±4.564^{a}	7.33±3.95^{a}	1.510	0.054

NIC: non-injected control; SHC: sham control; SHC: sham control; ZAC 3.64: 3.64 M Zn acetate; ZAC 5.23: 5.23 M Zn acetate.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 4 The effect of *in ovo* injection of zinc acetate (ZAC) on insulin-like growth factors (IGFs) and myogenic regulatory factors (MRFs) mRNA expression in muscle

Variable	Treatments				SEM	P-value
	NIC	SHC	ZAC 3.64	ZAC 5.23		
IGF-I	1.02±0.11^{a}	0.37±0.05^{b}	0.29±0.13^{b}	0.28±0.06^{b}	0.203	0.0002
IGF-II	1.01±0.08^{a}	1.36±0.23^{a}	2.86±1.41^{a}	2.82±1.76^{a}	0.567	0.055
IGF-R	1.05±0.17^{a}	0.7±0.23^{a}	0.7±0.25^{a}	0.5±0.21^{a}	0.110	0.4
MYOD	1.37±0.52^{b}	6.43±2.41^{b}	22.74±9.27^{a}	1.61±0.85^{b}	2.407	0.026
MYF-5	1.02±0.11^{b}	0.59±0.23^{b}	1.68±0.36^{b}	3.29±0.76^{a}	0.219	0.0048

NIC: non-injected control; SHC: sham control; SHC: sham control; ZAC 3.64: 3.64 M Zn acetate; ZAC 5.23: 5.23 M Zn acetate.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Hatchability, fertility and mortality

In this study, *in ovo* injection of ZAC significantly decreased hatchability (P<0.0001), increased early mortality (P<0.05) and there were no significant effect on fertility, middle and late mortality (P>0.05; Table 6).

Body and relative liver and muscle weight on day of hatch and body weight and feed conversion ratio during first week

ZAC *in ovo* injection had no significant effect on body weight and relative thigh weight on day of hatch (P>0.05). But ZAC significantly increased relative breast weight and liver weight (P<0.0001). Body weight and feed conversion ratio was significantly increased during first week (P<0.0001; Table 7).

IGFs and MRFs relative expression in embryo tissue

IGFs have important role on mammalian and avian embryo and fetus growth and development. Zn deficiency, decreased IGF-I mRNA expression but this changes did not completely inhibit the growth and Zn affected growth factors pathway that specifically direct cell proliferation (MacDonald, 2000). These pathways are mitogen-activated protein kinase (MAP kinase) and protein kinase C (PKC)

(MacDonald, 2000). The adding of physiological concentration of Zn to the cell increased MAP kinase activity and induced phosphorylation of cellular protein (MacDonald, 2000) and then increases proliferation and differentiation cells. PKC is a zinc-metalloenzyme (Hubbard *et al*. 1991).

In addition, Mccusker and Novakofski (2004) reported that the addition of physiological concentration of Zn depressed binding of IGF-I and IGF-II to IGFBP-5 and increased IGF-I and IGF-II binding to the IGF-IR on myoblasts. IGF-I and IGF-II enhanced proliferation and differentiation of myoblasts. In several studies, Zn supplementation in human and mice, increased expression of IGF-I in blood (Yu *et al*. 2005; Imamoglu *et al*. 2005; Hamza *et al*. 2012), this agrees with our results. But Akshat *et al*. (2012) reported Zn *in ovo* injection at the 14th day of incubation to not affect IGF-I expression in liver. Also, Wang *et al*. (2002) observed no significant effect on expression of this gene in the growth plate. The results of that study are different from our results. But increased levels of Zn in pigs diet, decreased IGF-I expression in muscle (Paulk *et al*. 2014).

Although, IGF-IR mRNA expression was decreased significantly by ZAC *in ovo* injection compared to non-injected control but it had no significant effect on the sham control. Probably, the day of injection and injection itself had negative effect on this gene expression. ZAC *in ovo* injection compared with non-injected control, decreased IGF-I expression. This result is similar to that reported by Paulk *et al*. (2014), who found that increasing the levels of Zn in diet of pigs, decreased IGF-R gene expression in muscle tissue.

Probably, down-regulation of IGF-IR with increased expression of IGF-1. Akshat *et al*. (2012) reported IGF-II mRNA expression was increased by Zn *in ovo* injection at 18th and 20th day of incubation and IGF-II in embryonic days affects on growth and development more than IGF-I. But our results pointed that Zn had no effect on IGF-II mRNA expression on 3th to 5th day of incubation (on day of injection to sampling).

MYOD and MYF-5 both played a role in the specification, formation and maintenance of myoblasts (Pownall *et al*. 2002; Richter *et al*. 2002; Parker *et al*. 2003). MYOD and MYF-5 is expressed in chicken embryo almost the same time (Lin-Jones and Hauschka, 1996). Zn deficiency in chick myoblasts culture decreased MYOD gene expression. So, adding Zn in this culture, increased MYOD (Petrie *et al*. 1996). As part of our research, ZAC 3.64 increased MYOD expression in embryo, but increased amount of ZAC (ZAC 5.23) is similar Paulk *et al*. (2014) research did not affect gene expression. In previous study (Paulk *et al*. 2014) and our study Zn did not affect MYF-5 expression.

In present study, *in ovo* injection of Zn increased IGF-I and MYOD expression during embryonic phase. Both of these genes are essential for proliferation and differentiation myoblast in during embryonic that IGF-I plays a role in regulating MYOD (Fernandez *et al*. 2002). IGF-I plays a multistep role, first acting upon myoblast proliferation, increasing the expression pattern of factors involved in the cell cycle progression, and then promoting myogenic differentiation by induction of myogenic regulatory factors such as MYOD (Richter *et al*. 2002). So 5.23 m*M* ZAC *in ovo* injection can increase IGF-I mRNA expression or ZAC may influence MYOD mRNA expression directly. On the other hand, increased IGF-I expression and ZAC had no effect on expression of MYF-5.

IGFs and MRFs relative expression in muscle (breast) tissue

MYOD and MYF-5 expression start during embryonic stage in myoblasts (Lin-Jones and Hauschka, 1996) for several days after hatch in breast muscle (Saitoh *et al*. 1993). In our study, the result of MYOD mRNA expression in breast muscle such as a result of this gene in embryo agrees with Petrie *et al*. (1996) results that the adding of Zn in chick myoblasts culture increased MYOD mRNA expression. Probably, Zn had direct effect on MYOD mRNA expression or other growth factors affecting this gene. Or the decreased IGF-I mRNA expression in muscle was not enough to change MYOD mRNA expression. MYF-5 mRNA expression in muscle was increased by ZAC, but in previous study was not affected by Zn (Paulk *et al*. 2014). Probably, myoblasts and muscle fibers were increased with increasing MYOD mRNA expression in embryo, and probably, after hatch hypertrophy was increased by injection Zn because MYOD and MYF-5 mRNA expression in muscle was increased by Zn injection. IGF-I and IGF-II increase myoblasts and Myofibers proliferation, differentiation and hypertrophy (Florini *et al*. 1996). *In ovo* injection of rh IGF-I at 3days of embryonic development increased skeletal muscle growth (Kocamis *et al*. 1998). Expression of IGF-II mRNA in muscle was similar to this gene expression in embryo in the present study. IGF-IR mRNA expression in muscle was not significantly influenced by Zn. This result did not agree with our results in embryo and Paulk *et al*. (2014) results. Paulk *et al*. (2014) showed increased Zn, decreased IGF-IR expression. IGF-I mRNA expression in muscle was similar to IGF-IR mRNA expression in embryo. This gene expression was decreased significantly by ZAC *in ovo* injection compared to non-injected control but not in the sham control. ZAC did not only changed IGF-I mRNA expression. Present results agree with experiments performed by Paulk *et al*. (2014) in muscle tissue.

Table 5 The effect of *in ovo* injection of zinc acetate (ZAC) on metallothionein (MT) on liver

Variable	Treatment				SEM	P-value
	NIC	SHC	ZAC 3.64	ZAC 5.23		
Metallothionein (MT)	1.25±0.5^{b}	3.73±0.05^{b}	7.88±2.70^{ab}	13.16±3.96^{a}	1.204	0.021

NIC: non-injected control; SHC: sham control; SHC: sham control; ZAC 3.64: 3.64 M Zn acetate; ZAC 5.23: 5.23 M Zn acetate.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 6 The effect of *in ovo* injection of zinc acetate (ZAC) on fertility, hatchability and embryonic mortality

Variable	Treatment				SEM	P-value
	NIC	SHC	ZAC 3.64	ZAC 5.23		
Fertility %	79.35±2.41^{a}	76.77±4.72^{a}	75.48±2.62^{a}	76.13±4.28^{a}	1.82	0.088
Hatchability of egg set %	61.94±6.32^{a}	52.90±5.73^{a}	34.84±4.26^{b}	27.74±2.62^{b}	2.47	0.0005
Hatchability of fertile %	77.68±6.41^{a}	69.62±7.59^{a}	46.05±5.21^{b}	36.32±2.46^{b}	2.86	0.0003
Early embryonic mortality %	5.01±1.57^{b}	19.08±4.22^{a}	28.11±5.29^{a}	27.04±6.61^{a}	2.39	0.0009
Middle embryonic mortality %	2.47±1.62^{a}	5.07±0.78^{a}	2.59±1.07^{a}	3.08±1.8^{a}	0.704	0.054
Late embryonic mortality %	0.00±0.00^{a}	3.30±1.51^{a}	5.91±2.22^{a}	6.61±3.61^{a}	1.12	0.192

NIC: non-injected control; SHC: sham control; SHC: sham control; ZAC 3.64: 3.64 M Zn acetate; ZAC 5.23: 5.23 M Zn acetate.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 7 The effect of *in ovo* injection of zinc acetate (ZAC) on body weight at hatch day (BW0), relative organ weight of body weight, body weight at 7 days after hatch (BW7) and feed conversion ratio (FCR)

Variable	Treatment				SEM	P-value
	NIC	SHC	ZAC 3.64	ZAC 5.23		
BW0	47.15±0.82^{a}	47.65±0.9^{a}	48.90±0.88^{a}	46.27±0.72^{a}	0.095	0.192
Relative breast weight %	2.86±0.09^{b}	2.74±0.08^{b}	3.68±0.08^{a}	3.50±0.06^{a}	0.039	< 0.0001
Relative thigh weight %	7.04±0.18^{a}	6.64±0.12^{a}	6.85±0.11^{a}	6.89±0.08^{a}	0.086	0.294
Relative liver weight %	2.04±0.8^{b}	2.58±0.09^{a}	2.64±0.08^{a}	2.76±0.23^{a}	0.087	< 0.0001
BW7	156.81±3.6^{b}	163.32±3.84^{b}	195.57±4.14^{a}	190.00±8.24^{a}	2.199	< 0.0001
FCR	1.52±0.05^{a}	1.69±0.06^{a}	0.9±0.03^{b}	1.25±0.09^{c}	0.030	< 0.0001

NIC: non-injected control; SHC: sham control; SHC: sham control; ZAC 3.64: 3.64 M Zn acetate; ZAC 5.23: 5.23 M Zn acetate.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Metallothionein relative expression in liver

Regulation, absorption, metabolism and storage of essential and non-essential trace elements have most important role of MT (Maret *et al*. 1998). *In ovo* injection of Zn in our study on the 3d day of incubation and previous study in different days of incubation (13th, 17th and 18th day of incubation) increased MT expression in liver (Fernando *et al*. 1989; Fleet and Mccormick, 1988) that in our study increased Zn levels, expression of MT mRNA was increased.

Hatchability, fertility and mortality

In some studies, increased Zn levels in diet of breeder, increased hatchability, fertility and decreased early and late embryonic mortality (Hudson *et al*. 2004; Favero *et al*. 2013). As well as Stanley *et al*. (2012) reported that Zn had no significant effect on hatchability but in the present study, *in ovo* injection of ZAC decreased hatchability and increased early embryonic mortality but no significant effect on fertility. These results agreed with experiments performed by Savage (1968), which stated that increased Zn in diet, decreased hatchability and increased early embryonic mortality. The interrelationship between copper and zinc has proven to affect hatchability, in which an excess of zinc will aggravate a copper deficiency.

Signs of a copper deficiency in the embryo include early mortality with anemia and a high incidence of hemorrhage following 3 to 4 days of incubation. Also, in study of Fernando *et al*. (1989), metal composition ($Zncl_2$ and $Cucl_2$) *in ovo* injection 4.8 mg per egg at 13 and 18 days of incubation, 80% of the embryo died within 10 hours after injection. Joshua *et al*. (2016) observed, *in ovo* feeding of nano minerals were not harmful to the developing embryo and did not influence the hatchability. In the present study, Zn *in ovo* injection decreased hatchability. Also, these treatments increased early embryonic mortality in sham control. It may be because of injection and amount of ZAC and day of *in ovo* injection (day 3 of incubation). Third day of incubation is one of the sensitive (critical) days and any stress in these days including *in ovo* injection, increased early embryonic mortality.

Body and relative organ weight on day of hatch and body weight and feed conversion ratio during first week

Zn is effective and essential element for growth and appetite regulation in avian (Batal *et al*. 2001). So that Zn deficiency in the diet, decrease the egg minerals, embryonic development, chick quality and livability (Blamberg *et al*. 1960; Richards and Steele, 1987; Caleb *et al*. 2006).

Hudson *et al*. (2004), added Zn in diet and observed no significant effect on body weight and organ weigh on hatch day that agrees our results. In our study, relative liver weight was increased in sham control and ZAC *in ovo* injection treatments. In Hudson *et al*. (2004) study, pipping muscle weight was not significantly different between treatments and was similar to relative thigh weight but relative breast weight has changed with ZAC. As noted, some of the genes related to growth and development, such as MYOD and MYF-5 expression in embryo and breast muscle at hatch day increased, which is the reason for the increased weight of this tissue.

Increased Zn levels in diets of broiler in some studies, significantly increased body weight and feed conversion ratio (Kucuk *et al*. 2003; Sahin *et al*. 2006; Norouzi *et al*. 2013). That is similar to our results in ZAC treatments. But other research reported that increased Zn in diet had no significant effect on body weight and feed conversion ratio (Saenmahayak *et al*. 2012; Ezzati *et al*. 2013; Shyam Sunder *et al*. 2013). Tako *et al*. (2005), injected zinc methionine in eggs at the 17th day of incubation, improved weight gain for 14 days after hatching. In the present study, we observed injected ZAC in eggs at 3 days of incubation, increased body weight after 7 days of hatching.

CONCLUSION

In conclusion, *in ovo* injection of ZAC was decreased hatchability and increased early embryo mortality. However it increased body weight at 7 days after hatching, relative breast and liver weight on hatching day and as well as MYOD, MYF-5 and IGF-I mRNA expression. Our results showed that the effect of Zn and levels of Zn in different tissues on similar genes are different. In this study, relative breast weight was increased due to increasing of IGF-I and MYOD mRNA expression in embryo and MYOD and MYF-5 mRNA expression in muscle. Although, Zn did not affected body weight on day of hatch but increased body weight during first week because MYOD and MYF-5 mRNA expression in muscle was increased by Zn injection that effect muscle hypertrophy after hatch. Decreasing IGF-I mRNA expression in muscle and increased body weight during first week and relative breast weight in hatching day.

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