Research in Veterinary Science 130 (2020) 87–92

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Supplementation of various zinc sources modify sexual development and testicular IGF family gene expression in pre-pubertal male Japanese quail

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ARTICLE INFO

Keywords:
IGF-I
Nanoparticle
Quail
Testis
Zinc-methionine

ABSTRACT

Zinc plays an important role in the regulation of insulin-like growth factor-I (IGF-I). IGF system, in turn, has a key role in the development and functions of the reproductive organs. This research was performed to investigate the effects of different sources of zinc on IGF-I gene expression and testicular development in pre-pubertal male Japanese quail. A total of 512 unsexed day-old Japanese quail chicks were randomly divided into 16 groups (4 dietary treatments × 4 replicates) and kept for 35 days. The control group diet was not supplemented with zinc whereas the diets of three groups were supplemented with 25 mg kg^{-1} zinc oxide (ZnO), zinc oxide nanoparticle (ZnON), and zinc-methionine (Zn-Met). On days 28 and 35, one birds from each subgroup were weighed, bled, and euthanized to evaluate gonado-somatic index (GSI), testicular histology, serum testosterone concentration, cloacal gland index (CGI), and the testicular IGF family gene expression. The results showed that GSI was higher in ZnON (2.307) than control (1.619) on day 35 ($P < .05$). Germinal epithelium thickness was higher in ZnON (78.88 μm) and Zn-Met (79.73 μm) than control (67.73 μm) on day 35 ($P < .05$). On day 35, the testosterone concentration was lowest in the control (5.830 ng/ml, $P < .05$). The CGI of 35-day-old birds was higher in Zn-Met (411.28) than the control (307.59, $P < .05$). IGF-IR mRNA expression was highest in Zn-Met group on day 28. Therefore, supplementation of diet with Zn-methionine is superior to other sources of zinc for diet supplementation in immature Japanese quail.

1. Introduction

Zinc is an essential micronutrient that plays a functional and structural role in a large number of macromolecules and enzymatic reactions (McCall et al., 2000; Jarosz et al., 2017). Zinc is a critical element for supporting the optimal levels of testosterone and testicle growth, consequently it is the most vital trace mineral for male sexual function (Egwurugwu et al., 2013). In addition, zinc has important roles in the nutritional regulation of insulin-like growth factor-I (IGF-I) (Starks, 2006). It has been evidenced that IGF, IGF-IR, and IGFBP genes are expressed in the testis (Baker et al., 1996). Moreover, IGF1 null male mice are infertile dwarfs that display a reduction of greater than 80% in both serum testosterone concentration and spermatogenesis (Baker et al., 1996).

Signaling of IGF-I is highly involved in regulating the final number of Sertoli cells during the pre-pubertal period (Pitetti et al., 2013). Differentiation of spermatogonia and Leydig cells (Yoon and Roser, 2010), regulation of spermatogonial DNA synthesis (Wang et al., 2014), and a direct/indirect enhancement of testosterone production in Leydig cells (Yoon and Roser, 2010) are other noteworthy functions of the IGF-I in testicular cells. Therefore, the IGF system significantly affects the development and functions of the reproductive organs (Fu et al., 2001).

The typical sources of zinc for feed do not always meet the requirement of animal (Attia et al., 2013). There is a growing concern about increasing the efficiency of dietary trace mineral in animals by replacing inorganic minerals with lower levels of organic chelated trace minerals (Bao et al., 2007). High concentrations in serum and liver, a high retention, and a low fecal excretion of zinc have been reported in the body when zinc was supplemented in organic form compared to inorganic form (Cao et al., 2002; Van Heugten et al., 2003). Zinc-methionine (Zn-Met) complex is transported from the intestinal lumen into mucosal cells, increasing tissue supply of zinc and thereby improving animal productivity (Mallaki et al., 2015). Additionally, supplementing diets with the Zn-Met source improves fertility parameters and

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https://doi.org/10.1016/j.rvsc.2020.03.004
Received 8 May 2019; Received in revised form 18 February 2020; Accepted 2 March 2020

hatchability traits in adult male Japanese quail (Khoobbakht et al., 2018).

Recently, ZnO (zinc oxide) nanoparticles have been used as a source of zinc for enhancing the zinc uptake (EI-Morshedi et al., 2014). It is generally accepted that nanoparticles more efficiently penetrate the cell membrane compared to bulk (Laurent et al., 2008). On the one hand, the requirement of Zn is higher for spermatogenesis, testicular growth and development than body growth (Arangasamy et al., 2018). This may be very important for Japanese quails as an avian that has acceptable growth performance. Nevertheless, there are few research studies to examine whether its inclusion as a feed additive would have beneficial effects on pre-pubertal male Japanese quails. The objective of the present study is to evaluate the effects of feeding different zinc sources (nano, organic, and inorganic) on gonadal morphometry, serum testosterone concentration, and the expression of IGF family gene in premature male Japanese quails.

2. Material and methods

2.1. Birds and diets

A total of 512 unsexed day-old Japanese quail chicks (*Coturnix coturnix japonica*, supplied from Hes Co.), with a body weight of 7.5 ± 3 g, were randomly divided into 16 groups of 32 chicks each (4 dietary treatments × 4 replicates). Animals cared for under experimental procedures and protocols approved by the veterinary organization of Iran. The basal corn-soybean meal diet was formulated to meet or exceed National Research Council (NRC, 1994) requirements for growing quails, except for zinc (Table 1). The basal diets were prepared by supplementing the Zn-free mineral premix. Quails in the control group were fed on a basal diet. Quails treated with zinc oxide (ZnO), zinc oxide nanoparticles (ZnON), and zinc methionine (Zn-Met) were fed with one of three additional diets prepared by adding 25 mg Zn /kg of ZnO or ZnONs (purchased from Nanosany Co.; purity: 99.9%; average particle size: 10–30 nm) or Zn-Met (ZINPRO). This study lasted 35 days and the chicks had free access to the diets and water. Chicks were maintained on standard lighting schedule in the floor pens. The temperature was 37 °C during the first day of age and then reduced by 0.5 °C/day until a temperature of 16–23 °C was reached at 4 weeks of age.

Table 1
Ingredients and chemical composition of the basal diet fed to Japanese quails.

Ingredient	%
Corn	48.3
Soybean meal	45
Vegetable Oil	3.2
Limestone	1.2
Dicalcium phosphate	1.2
Common salt	0.33
Vitamin premix[a]	0.25
Mineral premix[b]	0.25
DL-methionine	0.17
L-Lysine	0.1
Total	100.00
Chemical analyses, dry matter basis	
ME (kcal/kg)	2900
Crude protein (%)	24
Calcium (%)	0.85
Av. phosphorus (%)	0.38
Lysine (%)	1.35
Methionine + Cysteine (%)	0.8

[a] Vitamin premix in provided the following per kilogram of basal diet: A, 9000 IU; D_3, 2000 IU; E, 18 IU; K_3, 2 mg; B_1, 1.8 mg; B_2, 6.6 mg; B_3, 30 mg; B_6, 3 mg; B_7, 0.1 mg; B_{12}, 0.015 mg; choline chloride, 500 mg; Folic acid, 1 mg.

[b] Zinc-free trace mineral premix provided the following in milligrams per kilogram of basal diet: selenium, 0.2; manganese, 60; iron, 120; iodine, 0.3; copper, 5.

2.2. Sample collection and determination of gonado-somatic index

At 28 and 35 days of the trial, one randomly selected male birds from each pen replicate were weighed, bled individually, and euthanized. After dissecting, testes were weighted and the left testis was transferred to Bouin's solution for the histological examination and a part of the right testis was stored at −80 °C for gene expression evaluation. The weight of each testis was measured using an electronic analytical balance, followed by calculating the gonado-somatic index (100 × testis weight/body weight; Halldin et al., 2003).

2.3. Testicular histology and morphometry

To assess the histological changes, testicular tissues were dissected and samples were fixed in Bouin's fixative for 24 h. Next, the fixed testicular tissues were dehydrated, paraffin-embedded using an automatic tissue processor (ASP300, Leica, Wetzlar, Germany), and sectioned at 5 μm thick slices. The sections were stained with hematoxyline and eosin (H&E) and used for histological studies and morphometrical analysis. Afterward, the sections were viewed and photographed by a microscope (Magnum-3, Ceti, England) with an attached camera (Sony-DSC-H50). The evaluation of seminiferous tubular diameters (STD) and germinal epithelium thickness (GET) was performed by examining 20 fields in five histological sections from each testis using digitalized microscopic images (400×) with software Digimizer Version 4.1.1.0 (Med Calc Software, www.digimizer.com).

2.4. Assay of testosterone concentration and cloacal gland index (CGI)

Serum specimens were separated by centrifugation at 3000 rpm for 15 min at room temperature. Then, they were collected and frozen at −20 °C until the analysis. Serum concentrations of testosterone were evaluated in duplicate using a commercially available enzyme-linked immunoassay kit (DiaPlus Inc., USA). The reliably measure testosterone concentrations (analytical validation; parallelism and recovery) in the quail using a commercial ELISA kit was performed according to Gill et al., 2015. The sensitivity of the assay was 0.038 pg/ml and the intra- and inter-assay coefficients of variation were 6.5% and 5.9%, respectively. On the 35th day of the study, before euthanize, the width (lateral) and height (dorsoventral) of the cloacal gland of each bird were measured to the nearest 0.01 mm using a caliper. The result of height and width (mm^2) was used as an index for gland size (Biswas et al., 2013).

2.5. RNA extraction, cDNA synthesis, and real-time PCR conditions

The total RNA was extracted from the testicular tissue samples using Cinnagen RNA extraction kit (RNX-Plus Solution). RNA was reverse transcribed into cDNA using a High Capacity cDNA Archive Kit (Thermo SCIENTIFIC; No: #K1622) according to the manufacturer's instructions included in the kit. Relative quantification with real-time PCR was performed using Bio-Rad iQ5 Optical System (Bio-Rad Laboratories, Hercules, CA, USA). The primers were designed using Primer Premier 5.0 software based on GenBank sequence of target genes and listed in Table 2. According to the literature the β-actin gene was used as a housekeeping gene (Gasparino et al., 2013; Patel et al., 2019). The reactions were conducted in triplicate using the SYBR Green qPCR commercial kits (Bioron, Germany). The relative expression levels were expressed as $2^{-\triangle\triangle CT}$, and $\triangle$CT was calculated by subtracting CT (β-actin) from CT (target gene) (Livak and Schmittgen, 2001). The efficiency of the PCR amplification reaction was determined for each

Table 2
Primer pair sequences used for real-time PCR.

Gene	Sequence	Product size (bp)	Gen Bank accession number
IGF-I	F: 5´ AGACGCTTACACCACAAGGG3´ R: 5´ACGTACAGAGCGTGCAGATT3´	117	AF260131.1
IGF-IR	F: 5´ TCAAGACAGGAATACAAGA3´ R: 5´AATTATCAGATGGAGGAAG3´	177	AB292767.1
IGFBP2	F: 5´ AGGCCCATCACAACCATGAG3´ R: 5´ TGGGGATGTGGAGGGAGTAG3´	159	AF260701.1
Beta-Actin	F: 5´GGAAGTTACTCGCCTCTG3´ R: 5´AAAGACACTTGTTGGGTTAC3´	114	AB199913.1

Table 3
The effects of various zinc sources on Gonado-somatic index (%) of male Japanese quails.

Age (day)	Treatments (mean ± SE)				*p*-Value	F_{df}
	C	ZnO	ZnON	Zn-Met		
28	0.585^{b} ± 0.062	0.673^{ab} ± 0.070	0.886^{a} ± 0.065	0.805^{ab} ± 0.048	0.0217	$4.69_{3,12}$
35	1.619^{b} ± 0.038	1.751^{b} ± 0.091	2.307^{a} ± 0.167	2.055^{ab} ± 0.087	0.0027	$8.48_{3,12}$

Means in the same row with different superscripts differ (P < .05).

Table 4
The effects of various zinc sources on Japanese quail's testis histomorphometry.

Parameters (μm)	Age (day)	Treatments (mean ± SE)				p-Value	F_{df}
		C	ZnO	ZnON	Zn-Met		
STD*	28	192.23^{c} ± 1.855	198.41^{bc} ± 1.591	207.65^{a} ± 2.421	204.63^{ab} ± 2.521	0.0012	$10.32_{3,12}$
	35	248.55 ± 5.317	244.69 ± 4.542	254.20 ± 1.304	256.61 ± 1.928	0.1476	$2.15_{3,12}$
GET**	28	66.65 ± 0.565	68.61 ± 1.662	70.91 ± 2.073	71.07 ± 2.026	0.2559	$1.54_{3,12}$
	35	67.73^{b} ± 3.258	72.33^{ab} ± 1.59	78.88^{a} ± 3.207	79.73^{a} ± 1.203	0.0158	$5.19_{3,12}$

Means in the same row with different superscripts differ (*P* < .05).
* Seminiferous Tubular Diameter.
** Germinal Epithelium Thickness.

gene by performing standard curves consisting in serial dilutions of cDNA pool. Means of PCR efficiencies for IGF-I, IGF-IR, IGFBP2 and ß-actin were 1.91, 1.95, 1.86 and 1.98 respectively.

2.6. Statistical analysis

The data on 28 and 35 days were analyzed independently in completely randomized design with four treatments (zinc sources) and four replicates using the general linear model (GLM) procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC). The differences between treatment means were determined using the Tukey test. Probability values less than 0.05 (*P* < .05) were considered statistically significant.

3. Results

GSI changes during the development of male Japanese quails are presented in Table 3. GSI of the ZnON treatment was significantly higher in 28-day old quails as compared to the control group (*P* < .05). Moreover, GSI of 35-day-old birds in ZnON group was significantly (*P* < .05) higher than that of the quails that received diets supplemented with no Zn and ZnO. The effects of experimental diets on testis morphometric parameters (STD and GET) are shown in Table 4 and Fig. 1. The highest STD in 28-day-old quails was observed in the ZnON and Zn-Met groups as compared to the control group (*P* < .05). There were no statistically significant differences between the treatments in the STD on day 35 of the experiment (*P* > .05). GET in the 28-day old quails did not differ among the dietary treatments (*P* > .05). On day 35, birds receiving ZnON and Zn-Met had a significantly larger GET than the controls (*P* < .05).

The effects of different zinc sources on serum testosterone concentration of male quails are presented in Table 5. Testosterone concentrations 28 days and 35 days after treatment were significantly higher for birds on ZnON and Zn-Met diet (*P* < .05) than the controls. Furthermore, testosterone concentration in 35-day-old male quails fed with ZnO was significantly higher than that of the control (*P* < .05). The effects of dietary treatments on cloacal gland index are shown in Fig. 2. The cloacal gland index in 35-day-old quails fed Zn-Met was significantly (*P* < .05) higher than that of the quails that received diets supplemented with no Zn and ZnO.

The effects of dietary treatments on the IGF-I gene expression of quail testes are shown in Fig. 3A. IGF-I gene expression was markedly higher in ZnON and Zn-Met than the control and ZnO on the 28-day- and 35-day-old birds (*P* < .05). The effects of different zinc sources on quail testis IGFBP2 gene expression are illustrated in Fig. 3B. IGFBP2 gene expression at two stages was higher for birds on ZnON (*P* < .05) as compared to other treatments. The expression of IGF-IR mRNA in the Japanese quail testis in response to the different dietary sources of Zn is displayed in Fig. 3C. IGF-IR mRNA expression in the testis of 28-day-old quails fed with Zn-Met and ZnON showed significantly higher values compared to the controls (*P* < .05). The expression of IGF-IR mRNA 35 days after treatment was significantly greater in quails receiving Zn-Met and ZnON (*P* < .05) as compared to the control and ZnO groups.

4. Discussion

Zinc contributes to a wide variety of cellular processes as a cofactor for many enzymes (MacDonald, 2000). Hypogonadism is a major sign of zinc deficiency in both humans and animals (Karaca et al., 2007). Therefore, the supply of zinc in the diet is important for pre-pubertal

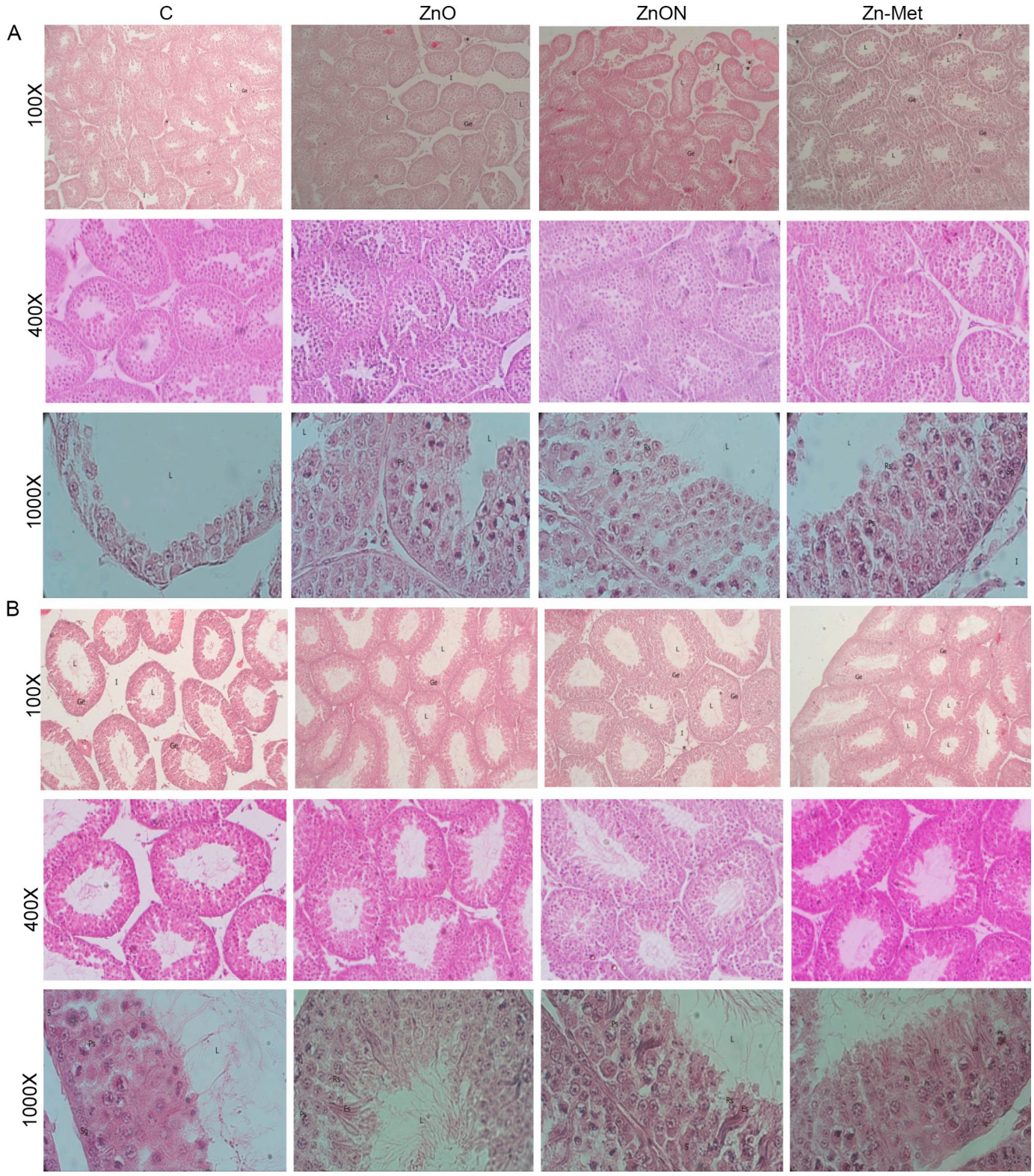

Fig. 1. Photomicrograph of testis histology in quail. C: control (without Zn), ZnO: Zinc oxide, ZnON: Zinc oxide nanoparticle and Zn-Met: Zinc- Methionine groups at 28 (A) and 35 (B) days of trial (H&E stained, Magnification 100, 400 and 1000×).
L: lumen, Ge: germinal epithelium, (*) represents Leydig cell, I: interstitial tissue, Ps: primary spermatocyte, S: Sertoli cell, Rs: Round spermatid, Es: Elongated spermatid.

animals. The results of this study showed that the serum testosterone concentrations were significantly higher in all groups that received different sources of zinc compared with the control group on day 35 while it was increased in ZnON and Zn-Met on day 28. Moreover, there was no statistically significant difference between Zn-Met and ZnON groups on cloacal gland index, but in ZnON and Zn-Met it was significantly higher than controls. Increase in level of testosterone is one of the first and important signs of male puberty. Zinc plays important roles

Table 5
The effects of various zinc sources on male quails testosterone concentrations (ng/ml).

Age (day)	Treatments (mean ± SE)				p-Value	F_{df}
	C	ZnO	ZnON	Zn-Met		
28	4.070^{b} ± 0.078	4.704^{b} ± 0.202	6.381^{a} ± 0.386	6.294^{a} ± 0.472	0.0020	$12.77_{3,12}$
35	5.830^{b} ± 0.558	7.710^{a} ± 0.102	7.734^{a} ± 0.187	7.792^{a} ± 0.113	0.0045	$9.95_{3,12}$

Means in the same row with different superscripts differ ($P < .05$).

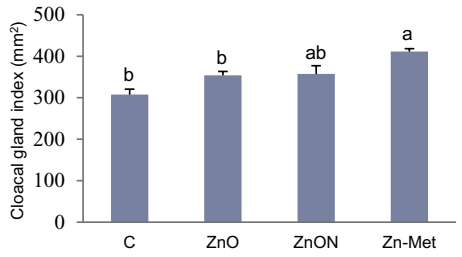

Fig. 2. The effects of various zinc sources on cloacal gland index (35 day old) of male Japanese quails. Different letters indicate significant differences ($P < .05$) between columns representing various groups of birds, whereas the same letters indicate a lack of differences between columns.

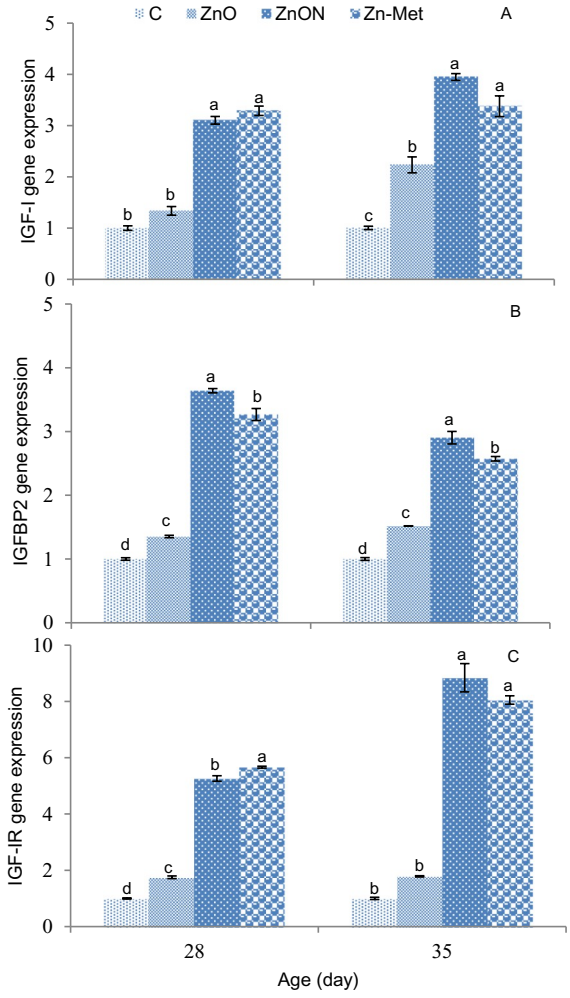

Fig. 3. The effects of various zinc sources on testis IGF-I (A), IGFBP2 (B) and IGF-IR (C) gene expression of Japanese quails. Different letters indicate significant differences ($P < .05$) between columns representing various groups of birds, whereas the same letters indicate a lack of differences between columns.

in the production and secretion of many sex hormones including testosterone and gonadotrophin-releasing hormone via activating the adenyl cyclase system (Babaei et al., 2007). It has been reported that zinc sources of greater bioavailability or high dietary levels of zinc can enhance the zinc concentration of the tissues (Yu et al., 2005). Furthermore, cloacal gland size and foam production are androgen-dependent and highly positively correlated with testes size as well as sexual activity in pre-pubertal Japanese quail (Sachs, 1967). In this regard, it has been reported that the size of the cloacal gland is a good marker of gonadal development in male quails (Ball and Balthazart, 2010). Overall, it appears that sexual development and the onset of puberty may be accelerated in birds of the Zn-Met and ZnON groups.

Gonado-somatic index was higher in ZnON group than control, although it was not significantly different in treatments receiving zinc. Moreover, microscopic evaluation of testis was showed that the diameter of the seminiferous tubules and the thickness of the germinal epithelium were higher in ZnON and Zn-Met groups than control on days 28 and 35 of the experiment, respectively. Therefore, it seems that supplementation of diet with ZnON and Zn-Met improve structural development of male gonad in pre-pubertal Japanese quail. Size changes of gonad associated with puberty are due to increased production of steroid hormones (Ruiz-Cortés, 2012). Inadequate zinc intake causes growth retardation, delayed sexual maturation, and infertility (Croxford et al., 2011). Besides, gonads are the most rapidly growing tissues in the body during maturation (Maret, 2009). It was reported that a reduction in testicular size was observed in bull calves fed a zinc-deficient diet (Kumar, 2003). Moreover, zinc deficiency can result in the reduction of testicular growth and seminiferous tubule shrinkage in young rams going through puberty (Goodarzi et al., 2017). The diameter of seminiferous tubules and thickness of the seminiferous epithelium have a positive relationship with seminiferous epithelium activity (Cheah and Yang, 2011). To our knowledge, organic zinc is absorbed in the small intestine actively (Mallaki et al., 2015). Moreover, intestinal zinc uptake in nanoform is more efficient than that in microform (Faiz et al., 2015). However, it seems that source of diet Zn plays an important role in the morpho-histological development of male gonad in pre-pubertal Japanese quail.

IGF plays an autocrine and/or paracrine action in the testis (Fu et al., 2001; Yoon and Roser, 2010). Signaling of IGF-I is highly involved in regulating the function and final number of Sertoli cells during the pre-pubertal period, which has a crucial role in the future male fertility (Pitetti et al., 2013). Our findings showed that IGF-I and IGF-IR mRNA expression of Japanese quail testis were elevated by consuming ZnON and Zn-Met on days 28 and 35; but, IGF-IR mRNA expression was highest in Zn-Met group on day 28. Moreover, male quails fed with Zn-methionine had the bigger cloacal gland index compared with the control. Consequently, organic zinc is superior to other sources of zinc for diet supplementation in pre-pubertal Japanese quail. It is reported that supplementation diet with Zn-Methionine upregulated the expression of IGF-I mRNA in mice liver (Starks, 2006). Adequate zinc intake can affect the gene expression of IGF family through transcription factors (Jackson et al., 2008). Zinc deficiency affects membrane signaling systems and intracellular second messengers that coordinate cell proliferation in response to IGF-I (MacDonald, 2000). It has been reported that the testes of IGF-I null mouse were

reduced in size. Moreover, it was found that they possessed a lower number of Leydig cells. According to this study, plasma testosterone concentrations were lower than the ones observed in wild mice (Griffeth et al., 2014). IGFs support spermatogenesis via the stimulating mitotic DNA synthesis in seminiferous tubules (Söder et al., 1992). Therefore, it is speculated that supplementation diet of pre-pubertal male quail with zinc-methionine may improve cell signaling involved the onset of puberty via an increase in expression of IGF family gene.

5. Conclusion

In conclusion, Zn-Met or ZnON may accelerate the bird sexual development and the onset of puberty presumably by improving the IGF family gene expression. Nevertheless, it seems that maturation of male Japanese quail was more efficiently accelerated by supplementation diet with Zinc-methionine.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

This research did not receive any specific funding. The authors express their thanks to the Hess-e Sabz-e Mandegar Co. for their virtual helps to performing this research.

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