Reduction of the negative effects of methionine on bone parameters in broilers' embryos by intra-egg injection of vitamin B_{12}

MOHAMMAD NASER NAZEM,[1] SHIMA TASHAROFI,[2] NEGIN AMIRI,[3] and SEPIDEH SABZEKAR[4]

[1] Assistant Professor of Anatomy, Histology and Embryology Department of Basic Sciences School of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran
[2] Department of Animal Science Researches, Agriculture and Natural Resources Education and Research Center of Kerman, Agriculture and Natural Resources Education and Research Organization of Iran
[3] PhD student of Animal Nutrition and Breeding, Department of Animal Science, Shahid Bahonar University of Kerman, Kerman, Iran
[4] Post graduate student of Veterinary Sciences, Shahid Bahonar University of Kerman, Kerman, Iran

Correspondence: shima.tasharrofi@gmail.com

Abstract

Methionine is a sulphur-containing amino acid. In addition to its general function as a component of proteins, methionine is crucial for a variety of methyltransferase reactions. Although, methionine is crucial for cartilage synthesis, its negative effects on bone diseases such as osteoporosis and tibial dyschondroplasia must not be ignored. These negative effects may be abrogated by vitamin B_{12} therapy. This study carried out to investigate the effects of intra-egg injection of some solutions including 1ml of distilled water, methionine, B_{12}, methionine with additional B_{12} on some bone and serum parameters in broilers' embryos (Ross 308). Fertile chick embryos were injected on day 4 of incubation into the yolk sac. On day 18, embryos were examined for left tibia parameters (amount of osteocyte and osteoblast cells and their length; the thickness of trabeculae and the collagen sediment) and serum parameters (amount of alkaline phosphatase and homocysteine). Methionine alone caused a loss of osteocytes length, osteoblasts heights and sedimentation of collagen, whereas those groups treated in addition with vitamin B_{12} were comparable to the control. Alkaline phosphatase concentration of serum in both B_{12} injected groups (B_{12} and B_{12}) was significantly upper than control. Homocysteine concentration of serum was higher in both methionine injected groups (methionine and B_{12}+methionine) compared with control. Data obtained in the current study suggest that supplementation with vitamin B_{12} may prevent defects in bone development parameters brought about by methionine.

Key words: broiler embryos, *in-ovo* injection, methionine, B_{12}.

Introduction

Although methionine is an essential amino acid and crucial for cartilage synthesis, it has some harmful effects on the age-related diseases, such as diabetes, obesity, osteoporosis and cancer.[1] So the potential to know and reduce its negative effects is increasing.[2-6] Extra dietary supplementation of methionine has adverse effects on bone matrix in broiler chickens and.[7-8] High-methionine-diets lead to high serum homocysteine levels which increases the risk of fracture.[9] Also methionine restriction shifts bone morphology due to delays in osteoblast differentiation.[6] However, some of the research considers the disadvantages effects of low-methionine-diet on bone parameters due to increased collagen degradation. In methionine-restricted mice, bone mass density and bone mineral content was elevated[5] and in methionine-restricted rat, bone mass decreased.[10] In addition, oral administration of methionine increased the growth of bones in rats.[11]

Furthermore vitamin B_{12} (cobalamin) is a fundamental cofactor in methionine metabolism.[12,13] B_{12} and methionine have direct and indirect relation to bone mass densitometry, respectively.[13] Use of B_{12} in diet decreases hip fracture in elders.[14,15] Deficiency of B_{12} in rats has no effect on the methylation reaction or on bone marrow folate levels, although the B_{12} concentration in bone marrow reduces in B_{12} deficiency.[16] In addition, no significant relation is found between the change of vertebral bone mineral density and B_{12} in women).[17]

Therefore in the current study, it was hypothesised that the injection of B_{12} into yolk sac at day 4 of incubation period, may rectify the adverse effects of methionine injection on embryos' bone parameters (measure the osteocyte and osteoblast cells and their length, and thickness of trabeculae in the left tibia) and serum metabolites (measure alkaline phosphatase and homocysteine).

Materials and method

Kerman (where the experiment was done) is located in the dry area with an average annual rainfall of 200 mm and maximum annual temperature of 40°C with altitude of 1500 to 2000m.

Design and Animals:

At day 4 of incubation, 40 fertile eggs (Mahan Farm, Kerman, Iran) from 35 week old broiler breeder chickens (Ross 308) were individually weighed and only eggs with a mean weight 57.5±1.2 g of those eggs weighed were randomly set on each of 4 treatments

1. Intra-egg injection of 1 ml distilled water (control).
2. Intra-egg injection of 1 ml distilled water containing 40 mg Methionine (Scharlau Co., Spain).
3. Intra-egg injection of 1 ml distilled water containing 100 µg vitamin B_{12} (Daroupakhsh Distribution Co., Iran).
4. Intra-egg injection of 1 ml distilled water containing 40 mg Methionine and 100 µg vitamin B_{12}) of 10 eggs each.

Incubators with automatic temperature control (37.5±0.1) and rotation of the eggs every 1 hour were maintained at 60% relative humidity until day 18 of incubation, when all eggs were opened to obtain the embryos for experiments.

In-Ovo Injection Procedure:

The injection site (broad end of the egg) was disinfected with 70 % alcohol (Ethanol) and then 1 ml of each solution was injected into the yolk sac at day 4 of incubation period, using a 22-gauge needle with depth of 28 mm from the broad end of the egg. The entry holes were sealed using melted paraffin wax.

Data collection

At 18th day of incubation period, all 40 eggs were weighed and opened and all embryos were dried, weighed and killed to measure alkaline phosphatase and homocysteine of serum and count the osteocyte and osteoblast cells and measure their lengths in the left tibia bone. The experimental protocols were reviewed and approved by the Animal Care Committee of Department of Basic Sciences School of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran.

For serum analysis, 18-day-old embryos were dried by soft tissues and decapitated to collect the blood in eppendorf tubes. Bloods then were centrifuged at 3000 x *g* for 10 min to gain serum. To measure the total homocysteine, homocysteine methyltransferase transfers the methyl group of d-methionine methylsulfonium to homocysteine, leading to the generation of l-methionine and d-methionine. Then d-amino acid oxidase oxidizes d-methionine with the simultaneous production of hydrogen peroxide to yield methylene blue (Diazyme Homocystein Enzymatic Colorimetric Assay Kit, Poway, CA 92064, USA) with an absorbance at 660 nm.[18] To measure alkaline phosphatase, biochemical kit (Pars Azmoon Co., Tehran, Iran) was used, following by use of a spectrophotometer at 405 nm.[19]

After killing at 18th day of incubation period, left tibias were taken from all embryos. Soft tissues were removed and tibias were used to determine the amount of osteocytes (in bone matrix) and their length, the amount of osteoblasts (in bone edges) and their lengths, and the thickness of trabeculae. In the first step, a cross section was taken from the centre of the tibia using a scalpel. The slices were fixed in 10% buffered formalin (100 ml of 40% formaldehyde, 4 g phosphate, 6.5 g dibasic sodium phosphate and 900 ml of distilled water) for 24 hrs and then the 10% buffered formalin was renewed. Tissues were dehydrated by transferring through a series of alcohols with increasing concentrations, placed into xylol and embedded in paraffin. Wax. Samples were cut into 5µm sections. Sections were then stained using Masson's trichrome (in order to evaluate the orientation and measurement the thickness of trabeculae and collagen sediment), von Kossa (in order to evaluate the calcium sediment on trabeculae) and haematoxylin-eosin (H&E) (for standard histopathological evaluation) methods and examined using light microscope. It is noticeable all sections of all groups that prepared for von Kossa method, were stained together. It is important to omit the colour concentration effect. On the Masson's trichrome stained slides, the mean of diameter of 30 trabeculae in each section was measured by using a digital system (Dino-eye, AM-7023, 5Mp, Taiwan). Counts of osteocyte and osteoblast cells, measurements of their lengths and heights and trabecular thicknesses were determined at a magnification of 40X.[20]

Statistical analysis

The data were analysed using the one way analysis of variance (ANOVA) of SPSS (16 Chicago, USA) software. Differences among treatments were determined with Tuki's test. Statements of significant probability were based on $p<0.05$.

Results

Body weight:

At 18-day-old embryos, bodyweight and egg weight/bodyweight were not significantly different between treatments ($P>0.05$) (Table 1).

Parameters	Treatments			
	Control	Methionine	B_{12}	Methionine+ B_{12}
18th day of incubation				
Body weight (g)	26.39±1.11	23.51±1.64	24.32±1.08	26.12±1.90
Egg weight/Body weight	2.20±0.10	2.45±0.07	2.36±0.19	2.20±0.09

Table 1. Effect of intra-egg injection of methionine and B_{12} on performance in broiler embryos.

Bone parameters:

Trabeculae in control group were formed normally. They were widely expanded from the cortex to medulla. In the methionine group, trabeculae were discontinued, very thin and developed weakly. B_{12} and methionine+ B_{12} groups showed similar aspects as controls (Figure 1).

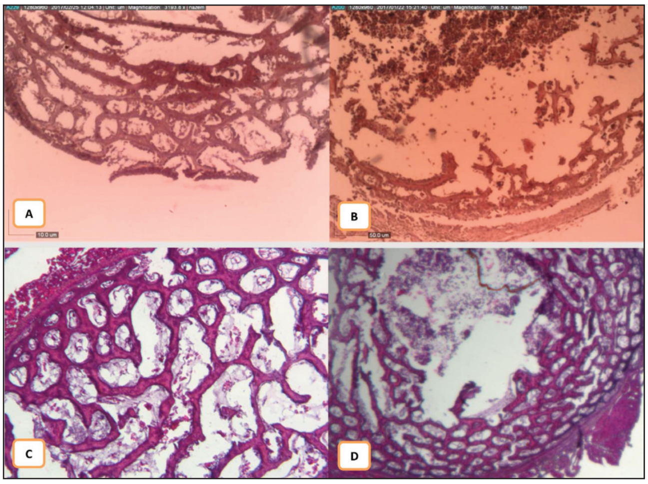

Figure 1. Trabecular formation in the tibia. The thickest trabeculae are seen in control (A), Vit B_{12} (C) and methionine+Vit B_{12} (D). The t in methionine group (B) are thin and discontinued (H&E, 40X).

Based on obtained results by Masson's trichrome method, collagen sediment was seen in all groups (Figure 2). On the other hand, calcium sediment was seen (brown to black) on all trabeculae by von Kossa method in all groups (Figure 3). In addition, based on Figure 3, obviously collagen sediment in methionine-injected group was less compared with Vit B_{12} and control.

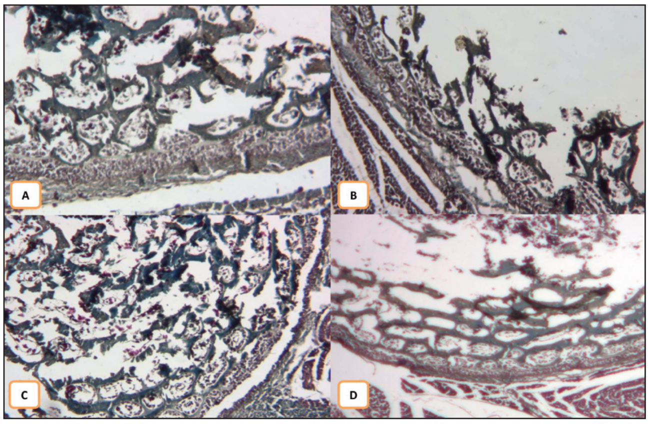

Figure 2. Collagen sediments are visible in the trabeculae of all groups by green colour (A: control, B: Methionine, C: Vit B_{12}, D: Methionine+Vit B_{12}; Masson's trichrome, 40X).

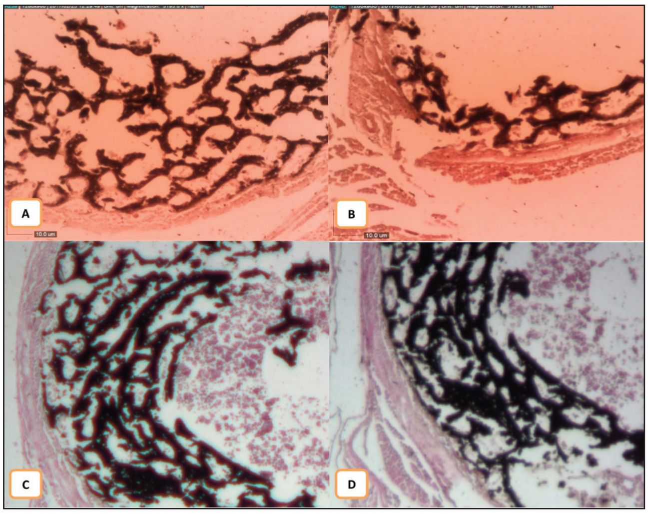

Figure 3. Calcium sedimentation in experimental groups (A: control, B: Methionine, C: Vit B_{12}, D: Methionine+Vit B_{12}). All groups show the presence of calcium (brown to black color) in trabeculae (von Kosaa, 40X).

According to Table 2, in the current study, amounts of osteocytes and osteoblasts did not change significantly by treatments ($P>0.05$), but numerally, intra-egg injection of methionine reduced both of them. Also methionine injection did negatively affect the length of osteocytes, height of osteoblasts and trabeculae thickness at 18th day of incubation period ($P<0.05$). Considerably, adding B_{12} to Methionine, rectified all of these bone parameters (length of osteocytes, height of osteoblasts and trabeculae thickness) in embryos ($P<0.05$).

Parameter	Treatments			
	Control	Methionine	B_{12}	Methionine+ B_{12}
Amount of osteocytes	3.80±0.37	3.00±0.32	3.70±0.21	4.00±0.15
Amount of osteoblasts	2.00±0.45	1.40±0.25	2.12±0.27	2.10±0.19
Length of osteocytes (µm)	3.78±0.07^{a}	3.49±0.06^{b}	4.18±0.22^{a}	4.20±0.18^{a}
Height of osteoblasts (µm)	3.26±0.11^{b}	2.80±0.12^{b}	4.10±0.22^{a}	4.14±0.27^{a}
Trabeculae thickness (µm)	10.37±0.34^{a}	9.50±0.26^{b}	10.41±0.12^{a}	10.52±0.10^{a}

^{a,b} Means within a row with different superscripts differ significantly (P<0.05).

Table 2. Effect of intra-egg injection of methionine and B_{12} on bone parameters in broiler embryos.

Serum parameters:

At 18th day of incubation, alkaline phosphatase of serum (Table 3) increased in both B_{12} groups (B_{12} and methionine+B_{12}) in comparison to control (P <0.05), and homocysteine of serum increased in both methionine groups (methionine and methionine+ B_{12}) (P<0.05).

Parameters	Treatments			
	Control	Methionine	B_{12}	Methionine+ B_{12}
Alkaline phosphatase of serum (µmol/L)	310.05±34.41^{b}	554.20±51.48^{ab}	798.92±38.41^{a}	822.38±62.41^{a}
Homocysteine of serum (µmol/L)	9.22±1.28^{b}	17.13±2.22^{a}	7.32±1.58^{b}	14.20±3.08^{a}

^{a,b} Means within a row with different superscripts differ significantly (P<0.05).

Table 3. Effect of intra-egg injection of methionine and B_{12} on serum parameters in broiler embryos.

Discussion

A large amount of studies have reported that *in-ovo* injection of different nutrients (specially carbohydrates, amino acids and proteins) into different sites of eggs (albumen, yolk sac and air sac) and at variable time of incubation, increases bodyweight at hatching, but all of these studies have injected the eggs at 18th day of incubation period, exactly the day we have broken the eggs to reach the embryos.[21-25]

About bone parameters, in contrast with our findings, decrease in bone density by methionine restriction in mice,[26,6] and ineffective results of methionine deficiency on bone marrow in rats are reported.[16] Bone development is influenced by hormones that are secreted either by cells within the tissue or in endocrine method.[6] Methionine restriction in diet of mice,[26] rabbit[27] and human[28] resulted in fallen levels of plasma adiponectin, and these low levels are known to reduction of osteoblast and osteoclast receptors of bone that leads to less bone improvements.[29] Also adiponectin receptors stimulate the secretion of alkaline phosphatase and osteocalcin hormones which are important in bone mineral density).[29] On the other hand, a lot of researchers demonstrate the negative impression of high levels of methionine on bone parameters which are in agreement with our results. High amounts of dietary sulphur amino acids lead to low thigh boneweight and density in rats.[8] Extra dietary supplementation of methionine has harmful effects on bone matrix[7] and causes tibial dyschondroplasia in broilers.[2]

One attractive hypothesis is that endocrine changes produced by the injection of methionine are really important in bone genesis. Because osteoblasts secrete collagen in bones, the proteins which are affective on osteoblasts performance are so important in bone formation.[30] For example, fibroblast growth factor (FGF) has basic role on differentiation of osteoblasts and every factor that influences FGF affects bones.[31] Extra FGF leads to defect in genesis of bones and access to more methionine terminates to more FGF.[32] However, FGF has different impression on differentiated or undifferentiated osteoblasts. In undifferentiated osteoblasts, FGF stops the differentiation.[31,32] Therefore, decrease of length of osteoblasts in methionine-injected group in the present study, is contributed to less collagen sediment in bones.

Osteocytes are responsible for calcification of bones and the tibial decrease in length of osteocytes and calcification are directly related to each other in our work.[30] Obviously, amount of osteocytes and their length are negatively impressed by methionine as shown in our study.[30]

Two fundamental enzymes in synthesis of methionine are methylene tetrahydrofolate reductase and methionine synthase reductase. Methylene tetrahydrofolate reductase gives the methyl for remethylation of homocysteine by B_{12}-dependent methionine synthase, thus deficiency of vitamin B_{12} can impair the remethylation of homocysteine.[33]

High concentration of protein (especially sulphur amino acids) in diet causes blood acidification finishes to bone's physical-chemical solubility and osteoporosis.[34-36]

So disorder in synthesis of collagen and less calcification in bones and high blood acidification and amount of homocysteine are four pivotal effects of extra access to methionine in animals.

Thus, like all other nutrients, both deficiency and extra contents of methionine could not terminate to good bone development. But because a higher dietary methionine concentration can improve growth performance in broilers, it is necessary to find a solution for its adverse effects on bone.[37,38] In the present study, supplying B_{12} via *in-ovo* injection procedure rectified bone parameters which were under disadvantage effects of extra methionine.

In conclusion, injecting methionine into yolk sac of fertile broiler eggs at 4th day of incubation adversely affects bone parameters of 18-day-old embryos but B_{12}-injection could reduce these bad effects. These results will allow us to validate intra egg injection of methionine+ B_{12} as an appropriate method not only for better performance but also for more developed bones.

References

1 **Vijiyan, V., Khandelwal, M., Manglani, K., Gupta, S.** and **Surolia, A.** (2013). Methionine down-regulates TLR4/MyD88/NF-κB signaling in osteoclast precursors to reduce bone loss during osteoporosis. *Br Pharm* 10(19): 156-158.

2 **Frankel, T.** (1995). Sulfate incorporation into organic bone matrix of the tibiotarsus of broiler chicks is reduced by excess dietary methionine. *Poult Sci* 74(3): 510-516.

3 **Komninou, D., Leutzinger, Y., Reddy, B.S.** and **Richie, J.P.** (2006). Methionine restriction inhibits colon carcinogenesis. *Nutr Cancer* 54, 202-208.

4 **Plaisance, E.P., Greenway, F.L., Boudreau, A., Hill, K.L., Johnson, W.D.** and **Krajcik, R.A.** (2011). Dietary methionine restriction increases fat oxidation in obese adults withmetabolic syndrome. *J Clin Endocrinol Metab* 96: 836-840.

5 **Ables, G.P., Perrone, C.E., Orentreich, D.** and **Orentreich, N.** (2012). Methionine-restricted C57BL/6J mice are resistant to diet-induced obesity and insulin resistance but have low bone density. *Plos One* 7:e51357.

6 **Ouattara, A., Cooke, D., Gopahakrishnan, R., Huang, T.,** and **Ables, G.P.** (2016). Methionine restriction alters bone morphology and affects osteoblast differentiation. *Bone Rep* 5: 33-42.

7 **Lohakare, J., Choi, J., Kim, J., Yong, J., Shim, Y., Hahn, T., & Chae, T**. (2005). Effects of dietary combinations of vitamin A, E and methionine on growth performance, meat quality and immunity in commercial broilers. *Asian-Aust J of Anim Sci* 18(4): 516-523.

8 **Whiting, S.** and **Draper, H.** (1981). Effect of a chronic acid load as sulfate or sulfur amino acids on bone metabolism in adult rats. *J Nutr* 111(10): 1721-1726.

9 **Saito, M.,** and **Fuji, K.** (2006). Degree of mineralization related collagen cross linking in the femoral neck cancellous bone in cases of hip fracture and controls. *Calcifi Tissue Int* 79: 160-168.

10 **Huang, T.H., Lewis J.L., Lin H.S., Kuo L.T., Mao S.W.** and **Tai, Y.S.** (2014). A methionine-restricted diet and endurance exercise decrease bone mass and extrinsic strength but increase intrinsic strength in growing male rats. *Nutr* 144: 621-630.

11 **McGrath, K.R.** and **Nakamoto, T.** (1985). Orally administered methionine alters the growth of tooth germs in newborn rats. *Ann Nutr Metab* 29(6): 374-380.

12 **Golbahar, J., Hamidi, A.** and **Aminzadeh, M.A.** (2004). Association of plasma folate, plasma total homocysteine but not methylene tetrahydrofolate reductase C6677 polymorphism with BMD in postmenopausal Iranian woman: a cross sectional study. *Bone* 35: 760-765.

13 **Morris, M.S.** and **Jacques, P.F.** (2005). Relation between Hcy and B-vitamins statues indicators and BMD in older Americans. *Bone* 37: 234-42.

14 **Sato, Y., Honda, Y., Iwamoto, J., Kanoko, T.** and **Satoh, K.** (2005). Effect of folate and metcobalamin on hip fractures in patients with stroke: a randomized controlled trial. *JAMA* 293: 1082-1088.

15 **McLean, R.R., Jacques, P.F.** and **Selhub, J.** (2008). Plasma B vitamins, homocysteine, and their relation with bone loss and hip fracture in elderly men and women. *J Clin Endocrinol Metab* 93: 2206-2212.

16 **Cheng, F.W., Shane, B.** and **Stokstad, E.L.R.** (1975). The Anti Folate Effect of Methionine on Bone Marrow of Normal and Vitamin B_{12} Deficient Rats. Available from: https://doi.org/10.1111/j.1365-2141.1975.tb00863.x

17 **Cagnassi, A., Bagni, B., Zini, A., Cannoletta, M., Generali, M.** and **Volpe, A**. (2008). Relation of folates, vitamin B12 and homocysteine to vertebral bone mineral density change in postmenopausal women, A five-year longitudinal evaluation. *Bone* 42: 314-320

18 **Matsuyama, N., Yamaguchi, M., Toyosato, M., Takayama, M.** and **Mizuno, K.** (2001). New enzymatic colorimetric assay for total homocysteine. *Cli Chem* 47(12): 2155-2157.

19 **Bar, J., Rosenberg, A.** and **Hurwitz, S.** (1982). Plasma and intestinal content of 1, 25 dihydroxy vitamin D3 in calcium or phosphorus restricted birds. Current Advances in Skeletogenesis. Pp197-200 in Proceeding of the 5th Workshop on Calcified Tissues. Elsevier Science Publishing, Amsterdam, The Netherlands.

20 **Zhao, W., Byrne, M.H., Wang, Y.** and **Krane, S.M.** (2000). Osteocyte and osteoblast apoptosis and excessive bone deposition accompany failure of collagenase cleavage of collagen. *J Clin Invest* 106(8): 941-949.

21 **Tako, E., Ferket, P.R.** and **Uni, Z.** (2004). Effects of in ovo feeding of carbohydrates and beta-hydroxy-beta-methylbutyrate on the development of chicken intestine. *Poult Sci* 83:2023-2028.

22 **Uni, Z., Ferket, P.R., Tako, E.** and **Kedar, O.** (2005). In ovo feeding improves energy status of late-term chicken embryos. *Poult Sci* 84:764-770.

23 **Uni, Z.** and **Ferket, R.P.** (2005). Methods for early nutrition and their potential. *World's Poult Sci J* 60:101-111.

24 **Chamani, M., Tasharofi, S., Foroudi, F., Sadeghi, A.A.** and **Aminafshar, M.** (2012). Evaluation the effects of in-ovo injection of different nutrients on hatch percentage, performance and carcass parameters of broilers. *Annals of Biol Res* 3(7): 3771-3776.

25 **Tasharofi, S., Mohammadi, F., Amiri, N.** and **Nazem, M.N.** (2018) Effects of intra-yolk-sac injection of dextrose and albumin on performance, jejunum morphology, liver and pectoral muscle glycogen and some serum metabolites of

broilers. *J of Anim Phys and Anim Nutr* 00:1-7. https://doi.org/10.1111/jpn.12882

26 **Li, M., Zhai, L., Wei, W.** and **Dong, J.** (2016). Effect of Methionine Restriction on Bone Density and NK Cell Activity. *Bio Med Res Int.* http://dx.doi.org/10.1155/2016/3571810

27 **Luo, E., Hu, J.** and **Bao, C.** (2012). Sustained release of adiponectin improves osteogenesis around hydroxyapatite implants by suppressing osteoclast activity in ovariectomized rabbits. *Acta Biomaterialia* 8(2): 734-743.

28 **Ozkurt, B., Ozkurt, Z.N., Altay, M., Aktekin, C.N., Glayan, O.C.** and **Tabak, Y.** (2009). The relationship between serum adiponectin level and anthropometry, bone mass, osteoporotic fracture risk in postmenopausal women. *J Bone Miner Metab* 20(2): 78-84.

29 **Shinoda, Y., Yamaguchi, M.** and **Ogata, N.** (2006). Regulation of bone formation by adiponectin through autocrine/paracrine and endocrine pathways. *J of Cell Biochem* 99(1): 196-208.

30 **Mescher Anthony, L.** (2013). Junqueira's basic histology text and atlas. 13th edition, McGraw Hill, New York; PP: 364-384.

31 **Mansukhani, A., Bellosta, P., Sahni, M.** and **Basilico, C.** (2000). Signaling by fibroblast growth factors (FGF) and fibroblast growth factor receptor 2 (FGFR2)-activating mutations blocks mineralization and induces apoptosis in osteoblasts. *J of Cell Biol* 149: 1297-1308.

32 **Raucci, A., Bellosta, P., Grassi, R., Basilico, C.** and **Mansukhani, A.** (2008). Osteoblast proliferation or differentiation is regulated by relative strengths of opposing signaling pathways. *J Cell Physiol* 215(2): 442-51.

33 **Lia, J.** and **Ward, R.L.** (2010). Four-folate and one-carbon metabolism and its impact on aberrant DNA methylation in cancer. *Adv in Genetics* 71: 79-121.

34 **Barzel, U.S.** and **Massey, L.K.** (1998). Excess Dietary protein can adversely affect bone. *J of Nutr* 128(6): 1051-1053.

35 **Bonjour, J.** (2005). Dietary protein, an essential nutrient for bone health. *J Am Coll Nutr* 24: 526S-36S.

36 **Heaney, R.P.** and **Layman, D.K.** (2008). Amount and type of protein influences bone health. *Am J of Cli Nutr* 87(5): 1567-1570.

37 **Ahmed, M.E.** and **Abbas, T.A.** (2011). Effects of Dietary Levels of Methionine on Broiler Performance and Carcass Characteristics. *Int J of Poult Sci* 10(2): 147-151.

38 **Chen, Y.P., Chen, X., Zhang, H.** and **Zhou, Y.M.** (2013). Effects of dietary concentrations of methionine on growth performance and oxidative status of broiler chickens with different hatching weight. *Bri Poult Sci* 54(4): 531-537.