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The Impact of Alpha-lipoic Acid Dietary Supplementation on Growth Performance, Liver and Bone Efficiency, and Expression Levels of Growth-Regulating Genes in Commercial Broilers

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ABSTRACT

Increasing bird growth is a crucial demand for all poultry producers. This occurs by the genetic improvement of the existing breeds and by improving the feeding management. The present study investigated the impact of Alpha-Lipoic Acid (ALA) supplementation in the diet on performance, serum parameters, tibia bone composition, and the expression levels of growth-related genes in chickens. A total of 120 day-old broiler chicks (Cobb 505) were used and divided into four groups. The control group was fed on a basal diet without the ALA supplement. The birds in groups of A50, A100, and A200 were fed on the formulated diet supplemented with ALA at doses of 50, 100, and 200 mg/kg of diet, respectively for 35 days. Results indicated that ALA supplementation significantly improved the birds' growth performance. This effect was associated with a marked upregulation of mRNA levels of *GHR* and *IGFR* and a significant downregulation of *MSTN* expression level. In addition, the ALA dietary provision caused a distinct improvement in liver function and bone efficiency. Thus, the improving effect of ALA on birds' growth performance is mediated by modulating the growth-regulating genes. In conclusion, ALA could be used as a good growth-promoter in dietary supplements.

Keywords: Alpha-lipoic Acid; Bone Efficiency; Broilers; Gene Expression; Growth Performance.

INTRODUCTION

Increasing bird's growth is a crucial demand for all poultry producers. This occurs by the genetic improvement of the existing breeds and by improving the feeding management (Petracci and Cavani, 2012). The latter is achieved through the dietary provision of feed additives such as antioxidants, enzymes, organic acids, probiotics, prebiotics and synbiotics along with herbal extracts to enhance the bird's growth performance and meat quality (Sohaib et al., 2018). Alpha-lipoic acid (ALA) is an effective multifunction feed additive and its use ranges from therapeutic applications to the dietary supplementations. It is widely dispensed in foods and has both water and fat-soluble properties thus it is absorbed from the diet (Packer et al., 1995). After absorption, ALA passes through cell membranes, leading to nutrient availability (Kofuji et al., 2008).

ALA plays an important role in energy metabolism as a result of its functions as a cofactor in many reactions that produce energy (Li et al., 2014). Thus, its dietary provision to farm animals, particularly broiler chickens, along with the cell produced it naturally in small quantity, directly scavenges free radicals and enhances fatty acid mobilization and energy expenditure. Therefore, it has a promoting effect on growth and the immune system, as well as decreases inflammation and oxidative stress (Sohaib et al., 2018). Recently, the application of ALA in broilers' diet is widespread to promote growth and improve the quality of carcass meat. It regulates the birds' growth performance by promoting energy metabolism and improving antioxidant status and immune response (Bai et al., 2012).

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ALA also protects liver of the broiler from damage as a result of chronic exposure to the low dose of aflatoxin B1 through improvement of plasma total protein, albumin, alkaline phosphatase, and the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Li et al., 2014). The aim of the present study was to evaluate the effect of ALA on performance, the liver and bone efficiency, and expression level of growth-related genes in chicken broilers.

MATERIALS AND METHODS

Ethical approval

The current study was approved by the Ethical Committee for live birds sampling at the Animal Health Research Institute, Egypt (License No. AHRI 35429).

Birds and experimental design

120 one-day-old chicks (Cobb-505 broiler strain) were used in the present study. Chicks were gained from a local farm and housed in the room. The room was cleaned and well-ventilated where the chicks were kept under good sanitation and hygienic management. The feed and water were available *ad libitum*. Chicks were allotted into four groups randomly with average body weight = 51.72±0.17 g/chick. For each treatment, 3 replicates contained 10 chicks were used. The C group (control one) was fed on a basal diet (Table 1). The basal diet was prepared according to broiler nutrition specification, 2007. The A50, A100, and A200 were fed on the formulated diet supplemented with ALA (Thiotacid® = It is an antioxidant made in EVA PHARMA Company, Egypt, in the form of tablets, each tablet contains 600 mg ALA) in a dose of 50, 100, and 200 mg/kg diet, respectively. All birds were weighed at the starting of the design and every week for five weeks while the diet was weighed every day to determine the feed intake and calculate the feed conversion ratio (FCR).

Sample collection and measurements of serum parameters

After 5 weeks, six birds from each group (2 birds/ replicate) were randomly selected and slaughtered to collect the blood samples. After the coagulation of blood, the serum samples were separated (centrifugation at 3000 rpm for 15 min) and kept at -20 °C. The serum was used to determine total protein, albumin, ALT, AST, and Alkaline phosphatase which were estimated using commercial kits (Bio-Diagnostic Company). While globulin was calculated by mathematical subtraction of albumin value from that of the total protein.

Measurement of tibia bone composition

After slaughtering, the left tibia bone of each slaughtered bird was isolated. The bones were dried in hot air oven at 60 °C for 48 hr to determine DM and moisture contents. The dried bones were finely ground and incinerated in the muffle furnace at 600 °C for 2 hr to determine ash content according to AOAC (2019). Calcium and phosphorus contents of tibia ash were determined by atomic absorption spectrometry.

Table 1. Ingredients and nutrients composition of the basal diets.

Items / Ingredients (%)	Diets		
	Starter (0-10 days)	**Grower (11-24 days)**	**Finisher (25 day-slaughter)**
Yellow Corn	58.0	63	66
Soybean meal (48%)	30.0	25	25
Corn gluten meal (60%)	6.1	6.1	2.5
Soy oil	1.5	2.1	3
Monocalcium phosphate[1]	1.85	1.6	1.5
Limestone[2]	1.12	0.95	0.9
Lysine[3]	0.45	0.35	0.2
DL-Methionine[4]	0.25	0.2	0.2
Common salt	0.43	0.4	0.4
Premix[1]	0.3	0.3	0.3
Nutrients composition			
ME (Kcal/Kg)	3084	3176	3215
Crude protein %	23.2	21.14	19.0
Lysine %	1.44	1.23	1.09
Methionine	0.68	0.6	0.54
Methionine & Cysteine	1.06	0.96	0.86
Calcium	1.08	0.91	0.87
Available phosphorus	0.5	0.45	0.42
Sodium	0.2	0.18	0.18

[1]Premix provides Vit A (12000 Iu), Vit D (5000 Iu), Vit E (50 mg), Vit K3 (3 mg), Vit B1 (3 mg), Vit B2 (8 mg), Vit B6 (4 mg), Vit B12 (0.016 mg), nicotinic acid (60 mg), pantothinic acid (15 mg), folic acid (2 mg), biotin (0.2 mg), iron (40 mg), copper (16 mg), zinc (100 mg), manganese (120 mg), iodine (1.25 mg), selenium (0.3 mg) per 1 kg diet.

Real-time polymerase chain reaction

Sample collection

From each treated group, six muscle samples (one sample/bird) were collected from the slaughtered birds and used for the gene expression analysis. The muscle samples were gathered into clean Eppendorf tubes, quickly frozen in liquid nitrogen then stored (-80 °C) until use.

Total RNA extraction and cDNA synthesis

Total RNA from muscle samples was extracted using easy RED total RNA extraction kits (Cat. No. 17063, Intron Biotechnology, Inc.) according to the manufacturer's instructions. Briefly, about 30 mg of muscle samples were ground into liquid nitrogen using a mortar and pestle. Then, 1 ml of easy RED and 200 µl chloroform were added, followed by centrifugation at

maximum speed (20817 xg). After that, RNA was pelleted and eluted in RNase free water (El-Kassas et al., 2016). RNA integrity was verified by agarose gel electrophoresis. A fixed concentration of RNA (2 µg) was reverse transcribed using the SensiFAST™ cDNA synthesis kit (Bioline, United Kingdom).

qRT-PCR assay

Specific primers (Table 2) were used to amplify *GHR*: growth hormone receptor, *IGF1R*: Receptor of insulin-like growth factor 1, and *MSTN*: Myostatin using the *β actin* as a housekeeping (internal standard) gene. The qPCR reaction mix, for each gene, contained 10 µl of SensiFast™ SYBR Lo-Rox master mix (Bioline, United Kingdom), 0.5 µM of each primer and 2 µl of cDNA. The qPCR assay for each tested gene was done in duplicate using Stratagene MX300P real-time PCR system (Agilent Technologies) with thermal cycling conditions were: initial denaturation at 95^{o}C for 15 minutes, followed by 40 cycles at 95^{o}C for 15 seconds, annealing for 1 minute at 60^{o}C for all genes. The dissociation curves were analyzed showing only one peak at a specific melting temperature for all tested genes indicating specifically amplified PCR products. The relative mRNA expression level for each gene was calculated using the $2^{-\Delta\Delta ct}$ method as described by Livak and Schmittgen (2001). In this context, the fold change for each gene was normalized against the house-keeping gene (*β actin*) and its comparable values of the control group (feeding basal diet without ALA supplementation).

Statistical analysis

The statistical analysis of data was performed using SPSS version 20**.** One-way ANOVA was used to test the effect of supplementing ALA into the birds' diet. The statistical significance at p-value < 0.05 between different supplemented groups was determined based on Duncan's test. The results were presented as mean ± SEM. For gene expression data, differences were considered to be statistically significant at p-values < 0.05

RESULTS

Growth performance

Statistical analysis of the data represented in Table (3) revealed that ALA supplementation (group A50, A100, and A200) significantly (p≤0.05) increase the final body weight, body weight gain, and average daily gain when compared with the control group. Also, statistical analysis of the FCR data indicated that the inclusion rate of ALA (group A50, A100, and A200) significantly (p≤0.05) improved FCR results when compared with the control group.

Serum liver function

Effect of dietary ALA supplementation on serum liver function of broiler chicken is presented in Table 4. Statistical analysis of the obtained result revealed that ALA supplementation (group A50, A100, and A200) significantly decreased (p≤0.05) serum ALT, AST, and AKP when compared with the control group. On the other hand, statistical analysis of the obtained data indicated that the inclusion rate of ALA (group A50, A100, and A200) significantly increased (p≤0.05) serum proteins when compared with the control group.

Tibia bone characteristics

Results of tibia bone analysis are shown in Table 5. Dietary supplementation of ALA (group A50, A100, and A200) significantly increased (P≤0.05) dry matter and ash contents in tibia bone of broiler chickens as compared to the control group. Broilers fed 100 mg ALA/kg diet significantly increased (P≤0.05) calcium concentration in tibia bone when compared with the control group. Moreover, there was no significant difference in phosphorus concentration in tibia bone among all groups.

Expression levels of growth-related gene

Supplementing ALA into the birds' diet significantly modified the relative mRNA transcript levels of *GHR*, *IGF1R*, and *MSTN* compared to their expressions in the case of birds fed basal diet (P = 0.009, P = 0.03, and P = 0.026, respectively). For *GHR* (Figure 1), ALA supplementation at 50 mg/kg diet stimulated a significant increase of *GHR* mRNA transcript levels (P = 0.002). Interestingly, increasing the level of ALA supplementation to 100 mg/kg diet significantly increased *GHR* gene expression level (P=0.014) but less than that in the case of 50 mg/kg diet supplementation. It resulted in an only 1.9-fold increase of the relative *GHR* gene expression level compared to 2.99-fold in the case of A 50 group. However, the ALA dietary provision at 200 mg/kg diet was able to markedly upregulate the *GHR* gene expression level (P=0.004). It resulted in 4.1-fold higher than that of non-supplemented birds (C).

For *IGF1R* gene expression (Figure 2), ALA stimulated a dose-dependent increase in *IGF1R* relative gene expression level. When it was added at 50 mg/kg diet, it induced a non-significant increase (2.03 fold) of *IGF1R* expression level. While, duplicating the ALA supplementing dose into the birds' diet to 100 and 200

mg/kg diet caused a distinct upregulation to *IGF1R* mRNA expression level (P=0.009 and P=0.013, respectively). It stimulated 3.58- and 3.74-fold increases of *IGF1R*, respectively. The *MSTN* mRNA copies were also modulated following ALA dietary provision (Figure 3). Its supplementation at 50 mg/kg diet significantly decreased the relative *MSTN* mRNA level (P<0.001). It resulted in 0.1-fold compared to the non-supplemented group (C). Also, ALA addition into birds' diet at 100 mg/kg diet resulted in a significant downregulation of *MSTN* gene expression level (P<0.001). It only caused 0.06-fold of *MSTN* mRNA copies. Additionally, birds fed ALA at 200 mg/kg diet showed a distinct reduction of *MSTN* expression level compared to those fed only basal diet without ALA supplementation (P<0.001). In summary, ALA dietary provision upregulated the gene expression levels of *IGF1R* and *GHR* genes and downregulated the *MSTN* mRNA level.

Table 2. Primer sequences used in qPCR analysis

Primer	Sequence	Reference
β-actin	Forward- 5' TACCTGAGCGCAAGTACTCTGCT 3' Reverse- 5' CATCGTACTCCTGCTTGCTGAT 3'	(El-Kassas et al., 2018)
IGF1R	Forward -5'GATCGGGCTTCACAACTT 3' Reverse -5'CCTTCGGAGGCTTATTTC 3'	(Chen et al., 2011)
MSTN	Forward or-5'GCAAAAGCTAGCAGTCTATG 3' Reverse -5' TCCGTCTTTTTCAGCGTTCT3'	(Dushyanth et al., 2016)
GHR	Forward - 5' AACACAGATACCCAACAGCC 3' Reverse - 5' AGAAGTCAGTGTTTGTCAGGG 3'	(Kamel et al., 2016)

IGF1R: Receptor of insulin like growth factor 1, *MSTN*: Myostatin, *GHR*: growth hormone receptor.

Table 3. Effect of dietary ALA on growth performance of broiler chickens.

Item	Control	A50	A100	A200
Initial weight (g)	51.79±0.56	52.06±0.63	51.49±0.69	51.62±0.57
Final weight (g)	1706.8±17.2 ^{b}	1753.2±14.0 ^{a}	1768.6±14.8 ^{a}	1754.8±11.8 ^{a}
Body weight gain (g)	1655.0±16.1 ^{b}	1701.1±13.2 ^{a}	1717.1±13.3 ^{a}	1703.2±10.5 ^{a}
Average daily gain (g)	47.29±0.46 ^{b}	48.6±0.38 ^{a}	49.06±0.38 ^{a}	48.66±0.30 ^{a}
Feed intake (g)	2961.8±6.1	2975.4±7.5	3002.4±9.2	2969.2±6.3
Feed conversion ratio	1.79±0.07 ^{b}	1.75 ±.09 ^{a}	1.75±0.05 ^{a}	1.74±0.03 ^{a}

ALA: alpha-lipoic acid. Control group received 0 mg ALA/kg diet, A50 group received 50mg ALA/kg diet, A100 group received 100mg ALA/kg diet, and A200 group received 200mg ALA/kg diet. Values are expressed as mean ± standard errors. Means with different superscript letters within the same row indicates significant difference at ($p \leq 0.05$).

Table 4. Serum liver function of broiler chicken supplemented with ALA at 35 days.

Parameters	Control	A50	A100	A200
ALT (u/l)	9.2± 0.416^{a}	7 ±0.577^{b}	6.667± 0.330^{b}	6.666± 0.882^{b}
AST (u/l)	185.2 ± 0.723^{a}	182.1± 0.493^{b}	181.667 ± 0.882^{b}	179.704 ± 0.788^{b}
AKP (Iu/l)	17.333 ±0.881^{a}	13.323 ±0.89^{b}	11.667 ±0.330^{bc}	11.000 ±0.577^{c}
Total protein (g/dl)	2.95 ± 0.029^{c}	3.700 ± 0.058^{b}	3.800 ± 0.057^{b}	4.125 ± 0.020^{a}
Albumin (g/dl)	1.33 ± 0.020^{c}	1.62 ± 0.017^{b}	1.510 ± 0.015^{c}	1.653 ± 0.044^{a}
Globulin (g/dl)	1.617 ± 0.009^{d}	2.080 ± 0.040^{c}	2.290 ±0.043^{b}	2.492 ±0.020^{a}

ALA: alpha-lipoic acid. ALT: alanine aminotransferase, AST: aspartate aminotransferase, AKP: alkaline phosphatase. Control group received 0 mg ALA/kg diet, A50 group received 50mg ALA/kg diet, A100 group received 100mg ALA/kg diet, and A200 group received 200mg ALA/kg diet. Values are expressed as mean ± standard errors. Means with different superscript letters within the same row indicates significant difference at ($p \leq 0.05$).

Table 5. Tibia bone composition of broiler chickens supplemented with ALA at 35 days.

Parameters	Control	A50	A100	A200
Dry matter %	40.98±0.05 ^{b}	45.21±0.33 ^{a}	44.10±0.33 ^{a}	44.0±0.19 ^{a}
Moisture %	59.02±.0.09 ^{a}	54.79±0.67 ^{b}	55.90±0.11 ^{b}	56.0±0. 45 ^{b}
Ash %	41.36±0.14 ^{b}	45.63±0.71 ^{a}	44.5±0.25 ^{a}	44.41±0.39 ^{a}
Ca %	34.04±1.84 ^{b}	37.32±0.33 ^{ab}	39.0±1.18 ^{a}	37.7±1.25 ^{ab}
P %	16.25±1.95	18.7±1.92	17.68±1.79	19.38±1.37

ALA: alpha-lipoic acid. Control group received 0 mg ALA/kg diet, A50 group received 50mg ALA/kg diet, A100 group received 100mg ALA/kg diet, and A200 group received 200mg ALA/kg diet. Values are expressed as mean ± standard errors. Means with different superscript letters within the same row indicates significant difference at ($p \leq 0.05$).

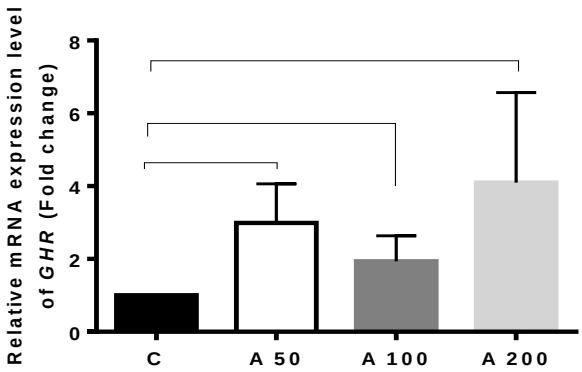

Figure 1. Relative mRNA expression level of *GHR* (fold change).C group received 0 mg alpha-lipoic acid (ALA)/kg diet, A50 group received 50mg ALA/kg diet, A100 group received 100mg ALA/kg diet, and A200 group received 200mg ALA/kg diet.

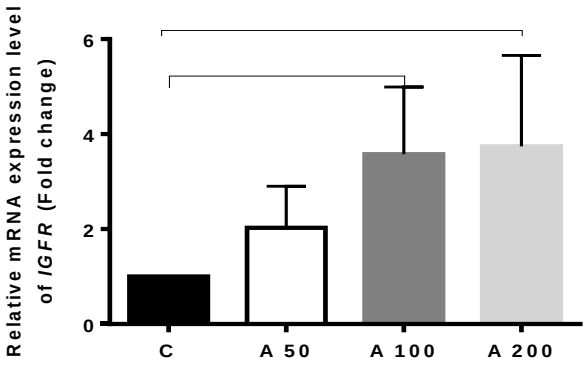

Figure 2. Relative mRNA expression level of *IGFR* (fold change). C group received 0 mg alpha-lipoic acid (ALA)/kg diet, A50 group received 50mg ALA/kg diet, A100 group received 100mg ALA/kg diet, and A200 group received 200mg ALA/kg diet.

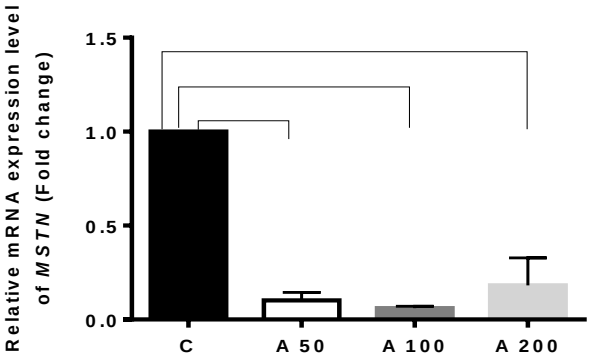

Figure 3. Relative mRNA expression level of *MSTN* (fold change). C group received 0 mg alpha-lipoic acid (ALA)/kg diet, A50 group received 50mg ALA/kg diet, A100 group received 100mg ALA/kg diet, and A200 group received 200mg ALA/kg diet.

DISCUSSION

Growth performance parameters significantly improved with dietary supplementation of ALA. These results may be attributed to the ability of the ALA to regulate energy metabolism where it is an integral component of mitochondria (Bai et al., 2012). Also, it has an antioxidant effect and acts as a coenzyme in carbohydrate metabolism in broilers (Packer et al., 2001). These results are consistent with findings of Guo et al. (2014) and Yoo et al. (2016) who reported improvement of birds' growth following ALA supplementation**.** Also, Lu et al. (2017) reported that ALA supplementation to broilers under ammonia stress could relieve stress status and restore production performance to normal levels. On the other hand, El-Senousey et al. (2013) and Zhang et al. (2014) reported that the supplementation of ALA to broiler's diet can lower weight gain and feed intake.

To deeply understand the mechanistic regulation of ALA to birds' growth, the relative mRNA levels of growth-regulating genes of *GHR, IGF1R,* and *MSTN* were measured for the first time in muscle tissues. Dietary supplementation of ALA significantly up-regulated the gene expression level of *GHR*, and *IGF1R*, while downregulated the mRNA level of *MSTN.* Modulating the expression level of these genes might explain the improved effect of the ALA on the birds' growth performance. Since, higher growth performance is positively correlated with higher levels of growth hormone and IGF1 (Wen et al., 2014). Thus, the upregulations of the gene expression level of *IGF1R* and *GHR* perhaps are a good confirmation of the improving effect of the ALA to birds' growth. Where, IGF-1 stimulates the birds' growth by increasing the rate of protein synthesis in the skeletal muscle **(**Boschiero et al., 2013). Consequently, the upregulation of *IGF1R* and *GHR* is often positively correlated with the increase in body weight following the ALA dietary supplementation. This effect might be explained by the increased levels of total protein, albumin, and globulin levels. On the other hand, ALA downregulated the *MSTN* gene expression level which probably is associated with the improved effects on growth performance. The myostatin which belongs to the transforming growth factor β (TGF-β) superfamily is a powerful negative regulator of muscle growth and differentiation (Jia et al., 2016). Thus, the higher expression of *MSTN* reduces the muscle fibers growth by downregulating myogenic differentiation factor (MyoD) and myogenic factor (Myf) expression level. Therefore,

the reduction of *MSTN* expression level following ALA dietary provision can explain the improving effect on growth performance.

In general, biochemical constituents of the serum reflect the health, nutrition, climate, and management conditions to which the animals are submitted (Minafra et al., 2010). The levels of biochemical parameters in the serum can be used as an indication of the productive performance of the birds and of metabolic diseases (Rotava et al., 2008). The liver injury could increase the concentrations of many serum enzymes such as AKP, AST, and ALT (Shanmugarajan et al., 2008) and decrease the concentration of total plasma proteins, as the liver is the organ that synthesizes proteins, especially albumin (Schmidt et al., 2007). In the present study, the result of biochemical parameters significantly improved at the different inclusion rates of ALA compared to the control group. This present finding is strongly supported by the work of Li et al. (2014). Disagree with the finding of Kim et al. (2015) who reported that the level and source of ALA didn't affect total protein, albumin, and globulin but decreased the liver enzymes in the serum. The results of this trial may be attributed to the role of ALA as a biological thiol antioxidant (Ahmad et al., 2018). Normally, free radicals produced in the body under normal physiological conditions and removed by antioxidants. The balance between antioxidant and free radicals negatively affected by sub-optimal diets and poor nutrient intakes or positively affected by dietary supplementation (Surai, 2007). Based on the result of liver function-related parameters it can be concluded that ALA supplemented in the diet at these levels has no bad effect on broilers.

The results of the present study showed the beneficial effect of ALA on bone efficiency as indicated by increasing ash and calcium contents in tibia bone. It is well known that there is a direct relationship between liver and kidney functions and bone efficiency through activation of vitamin D by hydroxylation (Koreleski and Swiatkiewicz, 2005). In the present study, ALA improved liver function as indicated by the reduction of serum ALT and AST enzymes. On the other hand, reactive oxygen species such as hydrogen peroxides, the hydroxyl group, and superoxide interact with nucleic acid altering cellular metabolism leading to oxidation of hepatocytes or accumulation of fat (Karaman et al., 2010), where activation of vitamin D takes place. ALA acts as an antioxidant that protects hepatocytes and renal cells against oxidative stress (Guo et al., 2014). This function was reflected in increasing calcium deposition in bone, subsequently increasing ash content and bone density. The best dose of ALA in the diets of broiler chickens increasing bone efficiency was 100 mg/ kg diet. Is there a direct relation between ALA and calcium deposition in bone? A question needs further investigation. More certainly, groups fed lipoic acid had significantly lower serum alkaline phosphatase activity than the control group. Decreasing the level of serum alkaline phosphatase activity reduced bone abnormalities and increased bone breaking strength (Ebrahimzadeh et al., 2013). From the literature, this study was the first one investigating the effect of dietary ALA supplementation on bone mineralization of broiler chickens.

CONCLUSION

In conclusion, ALA-supplemented diet resulted in significant improvements in the growth performance through regulating the liver functions, as well as growth-regulating genes and bone efficiency in broilers.

DECLARATIONS

Authors' contribution

Osama A. Sakr and Eldsoky Nassef prepared diet formula and measured growth parameters. Sabreen Ezzat Fadl measured serum biochemistry and made interpretation of the results. Seham El-Kassas measured gene expression and made interpretation of the results. Hazem Omar and Emad Waded helped in the measuring of serum biochemistry.

Conflicting interests

No conflict of interest

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