Kafkas Univ Vet Fak Derg
24 (6): 859-865, 2018
DOI: 10.9775/kvfd.2018.20103

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

Determination of the Stages of Deep Pectoral Myopathy Induced in Broilers Fed with Supplemental Coenzyme Q_{10} [1]

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[1] *This research has been financially supported by the Ministry of Education, Science and Technology Development of Republic of Serbia as a part of the Project No. TR 31033*

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Article Code: KVFD-2018-20103 Received: 11.05.2018 Accepted: 20.08.2018 Published Online: 20.08.2018

How to Cite This Article

Jojkic Z, Stojanovic S, Zikic D, Bjedov S, Milosevic V, Miler M, Uscebrka G: Determination of the stages of deep pectoral myopathy induced in broilers fed with supplemental coenzyme Q_{10}. *Kafkas Univ Vet Fak Derg,* 24 (6): 859-865, 2018. DOI: 10.9775/kvfd.2018.20103

Abstract

The aim of this study was to examine the structural characteristics and incidence of different stages of deep pectoral myopathy (DPM) that was induced in broilers fed a coenzyme Q_{10} (CoQ_{10}) supplemented diet. A total of 288 1-day-old chicks (Cobb 500) were equally divided among 8 pens (pens 1 to 8). The diet was the same for all chicks until day 35 post-hatching. Subsequently, broilers in pens 5 to 8 were fed the 20 mg of CoQ_{10}/kg finisher diet until the end of the experiment (day 42 post-hatching). To induce DPM, 5 male birds from each of the pens 1 to 8 were subjected to encouraged wing flapping (EWF) at the end of their 37th day. At the end of the trial, the incidence of DPM stages in broilers was determined and an analysis of the histological parameters of deep pectoral muscles was performed. Results showed that, in the groups subjected to EWF, broilers with the CoQ_{10} supplement had a lower average DPM stage and volume density of necrotic muscle cells, as well as a higher volume density of non-necrotic muscle cells. These results can be related to the antioxidant properties of CoQ_{10}, which, in chickens subjected to EWF, reduced the effects of DPM on cell necrosis and muscle tissue damage.

Keywords: *Deep pectoral myopathy, Coenzyme Q_{10}, Broiler*

İlave Koenzim Q_{10} İle Beslenerek Derin Pektoral Myopati Oluşturulan Broiler Piliçlerde Miyopatinin Devrelerinin Belirlenmesi

Öz

Bu çalışmanın amacı; koenzim Q_{10} (CoQ_{10}) ilaveli diyet ile beslenerek derin pektoral myopati (DPM) oluşturulan broiler piliçte meydana gelen myopatinin farklı devrelerini ve yapısal özelliklerini araştırmaktır. Toplam 288 adet 1 günlük civciv (Cobb 500) 8 kafese eşit olarak dağıtıldı. Tüm civcivler için yumurtadan çıkmayı takiben 35. güne kadar diyet aynı tutuldu. Takibinde, 5'den 8'e kadar olan kafeslerdeki civcivler çalışmanın son gününe kadar (yumurtadan çıkmayı takiben 42. gün) 20 mg CoQ_{10}/kg bitirme diyeti ile beslendi. DPM oluşturmak amacıyla, 1'den 8'e kadar olan kafeslerin her birinde 5 erkek civciv 37. günlerinde kanat çırpmaya zorlandı. Deneme sonunda, broiler piliçlerdeki DPM devreleri belirlendi ve derin pektoral kaslarda histolojik parametreler analiz edildi. Elde edilen sonuçlar kanat çırpmaya zorlanan gruplarda CoQ_{10} ilavesi ile beslenenlerde daha az DPM devresi ve daha az nekrotik kas hücresi hacim yoğunluğu ile daha fazla nekrotik olmayan kas hücresi yoğunluğunun oluştuğunu gösterdi. Bu sonuçlar CoQ_{10}'nın antioksidan özellikleri ile ilişkili olup, kanat çırpmaya zorlanan piliçlerde DPM'nin hücre nekrozu ve kas dokusu hasarı etkilerini azaltabilir.

Anahtar sözcükler: *Derin pektoral myopati, Koenzim Q_{10}, Broiler*

INTRODUCTION

Several myopathies which occur in broilers, such as deep pectoral myopathy (DPM), white striping (WS), and wooden breast (WB), are thought to be related to modern trends in poultry production that target increased growth rate,

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body weight and muscle mass of chickens.While WS is characterized by the occurrence of white striations [1], and WB by hardening of the breast muscle [2], DPM is associated with ischemic necrosis ofthe deep pectoral muscles [3].

The function of the deep pectoral muscle is related to wing activity [4]; its location near the sternum, the non-elastic fascia which surround the muscle, and the great size of the breasts in broiler chickens, leave insufficient space for the muscle to expand in response to wing movements. This leads to increased pressure within the muscle, blood flow obstruction and inadequate oxygen supply [5]. Tissue necrosis occurs as a result of ischemia. Described processes represent main characteristics of DPM.

Among several types of stresses (technological, environmental, nutritional, and internal) that can occur in poultry production [6], some of them caused by, for example, increased stocking density, cathcing or carrying the chickens by their wings, inappropriate weighing methods, etc., could result in unnecessary wing movements. This contributes to the development of more or less pronounced degrees of DPM [7]. In recent studies, methods defined as forced wing flapping [8] and encouraged wing flapping [9] have been used for the induction and examination of DPM in broilers.

Oxidative stress is related to elevated production of reactive oxygen species [10,11] that are associated with tissue necrosis [12]. Oxidative stress can be induced during hypoxia [13,14]. Considering that hypoxia is one of the characteristics of DPM (hypoxia can be induced through the occlusion of blood vessels and ischemia), it is therefore important to examine the influence of antioxidants on DPM development.

Beside the antioxidant properties of coenzyme Q_{10} (CoQ_{10}) [15], it has an important role in oxidative phosphorylation and ATP synthesis [16]. CoQ_{10} is already used as a supplement in broiler diets for the treatment of certain diseases and to improve immune functions. Its supplementation has been found to decrease mortality due to pulmonary hypertension syndrome in broilers exposed to low ambient temperature [17]. Furthermore, in research on Newcastle disease, chicks antibody titer was higher on day 21 post-hatching in broilers supplemented with 20 mg of CoQ_{10}/kg of diet [18].

The purpose of this study was to identify and examine the stages of DPM induced in broilers fed with supplemental CoQ_{10}.

MATERIAL and METHODS

Animals and Experimental Design

For the purpose of this study, 288 1-day-old chicks (Cobb 500) were randomly distributed between 8 floor pens (pens 1 to 8), 36 chicks in each pen. Pens 1 to 4 represented 4 replicates, where chickens were fed with starter (days 1-14), grower (days 15-35) and finisher (days 36-42) diets *(Table 1)*. Meanwhile pens 5 to 8 represented 4 replicates where feeding programs were the same as in pens 1 to 4, but with the additional supplementation of 20 mg of CoQ_{10}/kg finisher diet (days 36-42). To induce DPM, 5 male birds from each of the pens 1 to 4, as well as from each of the pens 5 to 8, with a body weight nearest to the mean body weight of the male birds, were subjected to encouraged wing flapping (EWF) at the end of day 37 post-hatching.

The chickens of the control group were fed a basal diet, while the 3 treatments included: 1) chickens fed the basal diet and subjected to EWF; 2) chickens fed a basal diet with CoQ_{10} supplementation; and 3) chickens fed a basal diet supplemented with CoQ_{10} that were subjected to EWF. Food and water were available *ad libitum*. The use of animals in this experiment was approved by the decision of the Ministry of Education, Science and Technological Development of Serbia (Decision No. 401-00-9/2011 of 25 January 2011).

For DPM induction in broiler chickens, the EWF method as described by Lien et al.[9] was applied. This method was basically performed in several steps. First, the chicken was held in the palms of the hands at a height of 1 m, after

Table 1. *Composition of experimental diets*

Ingredients	Starter (0-14 d)	Grower (15-35 d)	Finisher (36-42 d)
Diet composition (g/kg)			
Corn	488.0	528.0	553.0
Full fat soybean	170.0	180.0	165.0
Soybean meal (44% CP)	270.0	220.0	200.0
Soy oil	20.0	20.0	30.0
Monocalcium phosphate	15.0	15.0	15.0
Limestone	18.0	18.0	18.0
Salt	2.5	2.5	2.5
Sodium bicarbonate	3.0	3.0	3.0
L-Lysine HCl	1.0	1.0	1.0
DL-methionine	2.5	2.5	2.5
Vitamin and mineral premix	10	10	10
Calculated nutrient composition			
AME (MJ/kg)	12.87	13.11	13.43
Crude protein (g/kg)	225.5	210.6	198.4
Lysine (g/kg)	13.4	12.4	11.5
Methionine (g/kg)	5.9	5.7	5.6
Ca (g/kg)	11.5	11.4	11.3
P total (g/kg)	7.5	7.3	7.2
P available (g/kg)	4.4	4.3	4.2

CP: crude protein, AME: apparent metabolizable energy

which it was raised to a height of 2 m. From that height, each chicken was allowed to fall freely back to a height of 1 m. Flapping of the chicken's wings occurred during the fall. One cycle of EWF consisted of the previously described procedure. In our study, broilers were treated with EWF at the end of day 37 post-hatching, with a total treatment duration of 45 seconds, which included about 20 cycles of EWF.

During the rearing period, which lasted 42 days, body weight of broilers was in accordance with performance objectives for Cobb 500, and there were no significant differences between treatments. Subsequently, the 5 male birds from each of the pens 1 to 8 that were subjected to EWF were sacrificed. From each of the pens 1 to 4, as well as from each of the pens 5 to 8, 5 male birds that were not subjected to EWF, with body weights nearest to the mean body weight of the male birds, were also sacrificed.

Detection of DPM Stages

In order to reach the deep pectoral muscle, a ventral cut was made through the breast tissue. A score of DPM 0 was used to indicate no signs of myopathy in deep pectoral muscles. Categorization of DPM into a further 4 stages was made according to Kijowski and Kupinska [8]. Hemorrhages and clotted blood in the vessels characterized stage 1 (DPM 1). In stage 2 (DPM 2), necrosis and fibrosis of muscle tissue were present, while the color of the deep pectoral muscle was pale pink. Stage 3 (DPM 3) was related to a change in the color of muscle tissue to green, particulary in the central muscle area. A high degree of muscle tissue necrosis characterized stage 4 (DPM 4), which was manifested by an intensive green color of muscle, which in some parts turned to white and gray.

Morphometric and Stereological Examinations

For the purpose of histological analysis, samples of deep pectoral muscle *(M. supracoracoideus)* were taken after determination of macroscopic DPM stage. Muscle tissue samples were fixed in a 10% buffered formalin solution, followed by several stages of tissue processing: dehydration, clearing, and embedding in paraffin [19,20]. Then, the samples were cut into serial 5µm thick sections using a microtome. Hematoxylin-eosin (H&E) and the Mallory trichrome staining method were performed [21,22] to observe the following parameters: diameter and volume density of non-necrotic and necrotic muscle cells, diameter and volume density of adipose cells, and volume density of connective tissue proper cells.

Samples were analyzed by light microscopy using a Leica DMLS microscope with a Leica DC 300 digital camera and Leica IM 1000 software (Leica Imaging Systems Ltd, Cambridge, UK). The diameter of muscle as well as of adipose cells was measured as the average of the longest lines drawn across the length and width of their cross-sections. The M42 multipurpose testing system was used to measure the volume density of certain cells (muscle cells, adipose cells, and connective tissue proper cells). This testing system represents a grid that consists of 21 straight-line segments, while the test points are located at both ends of each line, making 42 test points in total [23,24]. To calculate the volume density of certain cells, the following formula was used:

$$Vv(c) = \frac{P(c)}{P(m)} \cdot 100\ (\%)$$

where Vv(c) is the volume density of certain cells, P(c) is the number of test points lying over the certain cells, and P(m) is the number of test points lying over the muscle.

Statistical Analysis

Analysis of variance (ANOVA) and *post hoc* Tukey's test were used to determinate the influence of different treatments on the value of the observed histological parameters. To study the influence of different treatments on the incidence of DPM stages, chi-square test followed by the Bonferroni correction was used; a significance level of 0.05 was applied. Statistical tests were carried out using the software package Statistica 13.0 (Dell Software, Round Rock, Texas, USA).

RESULTS

Measurements showed that in those groups where chickens were fed a basal diet and treated with EWF, the incidence of DPM stage 0 was lower ($P<0.01$), while the incidence of DPM stage 3 as well as average DPM stage was higher ($P<0.01$) compared to groups without EWF treatment *(Table 2)*. Between groups with EWF treatment, average DPM stage was lower ($P<0.05$) in groupswhere chickens were fed a basal diet with CoQ_{10} supplementation.

All stages of DPM were detected during the examination of deep pectoral muscles in sacrificed birds *(Fig. 1)*. Deep pectoral muscles with no signs of DPM were labeled as DPM 0. Microscopically these muscles showed standard characteristics of skeletal muscles. DPM stage 1, characterized by red colored deep pectoral muscle with excessive fluid in the damaged area, histologically showed hyperemia, edema, and numerous leucocytes. DPM stage 2 was defined macroscopically by a pale plum color of the deep pectoral muscle with an accompanying fibrotic texture, and microscopically by structural alterations such as necrosis of muscle cells with pale cytoplasm and nuclei. Certain muscle cells were swollen during the early stages of necrosis. In DPM stage 3, muscle samples taken from the central, green parts of the deep pectoral muscle, histologically demonstrated an increased quantity of fibrous tissue with the occasional presence of adipose cells. Muscle tissue classed as DPM stage 4 macroscopically

Table 2. *Effects of treatments on the incidence of deep pectoral myopathy (DPM) stages in broilers at 42 days post-hatching*

Item	Groups			
	Control	Basal Diet with EWF Treatment	CoQ_{10} Supplementation	CoQ_{10} Supplementation with EWF Treatment
DPM 0 (%)	87.50^{A}	37.50^{B}	87.50^{A}	62.50^{AB}
DPM 1 (%)	6.25	6.25	12.50	18.75
DPM 2 (%)	6.25	12.50	0	6.25
DPM 3 (%)	0^{B}	37.50^{A}	0^{B}	12.50^{AB}
DPM 4 (%)	0	6.25	0	0
Average DPM stage	0.19^{Bab}	1.69^{Aa}	0.12^{Bab}	0.69^{ABb}

EWF: encouraged wing flapping. CoQ_{10}: Coenzyme Q_{10}
^{A,B} Values in the same row without a common superscript capital letter differ significantly (P<0.01)
^{a,b} Values in the same row without a common superscript lowercase letter differ significantly (P<0.05)

Table 3. *Effects of treatments on histological parameters of muscle, adipose, and proper connective tissue of M. supracoracoideus of broilers at 42 days post-hatching*

Parameter	Groups			
	Control	Basal Diet with EWF Treatment	CoQ_{10} Supplementation	CoQ_{10} Supplementation with EWF Treatment
Muscle tissue				
Diameter of non-necrotic muscle cell (µm)	62.06±1.86	56.40±1.66	62.17±1.47	60.10±1.90
Diameter of necrotic muscle cell (µm)	54.23±3.27	50.99±2.78	55.24±3.86	52.05±2.99
Volume density of non-necrotic muscle cells (%)	$82.05±0.70^{A}$	$47.61±1.00^{C}$	$83.64±0.77^{A}$	$64.16±1.28^{B}$
Volume density of necrotic muscle cells (%)	$0.68±0.15^{C}$	$21.55±0.98^{A}$	$0.62±0.18^{C}$	$8.79±0.84^{B}$
Adipose tissue				
Diameter of adipose cell (µm)	24.74±1.75	19.07±1.68	22.61±0.56	20.45 ±1.73
Volume density of adipose cells (%)	$0.36±0.1^{B}$	$3.05±0.44^{A}$	$0.42±0.1^{B}$	$2.54±0.33^{A}$
Proper connective tissue				
Volume density of proper connective tissuecells (%)	$12.30±0.45^{B}$	$22.36±0.57^{A}$	$11.36±0.41^{B}$	$20.97±0.67^{A}$

Values are presented as mean ± standard error of the mean
EWF: encouraged wing flapping. CoQ_{10}: Coenzyme Q_{10}
^{A,B} Values in the same row without a common superscript capital letter differ significantly (P<0.01)

contained whitish regions within areas of green necrotic tissue; in addition, partial replacement of damaged muscle tissue with fibrous tissue and in places with adipose tissue, were detected at the microscopic level.

Histological examination of muscle tissue showed that, in groups with EWF treatment, volume density of necrotic muscle cells was higher (P<0.01), while the volume density of non-necrotic muscle cells was lower (P<0.01) compared to other groups *(Table 3)*. Between groups with EWF treatment, volume density of necrotic muscle cells was higher (P<0.01), while volume density of non-necrotic muscle cells was lower (P<0.01) in groups fed the basal diet. Microscopic observations of adipose and connective tissue showed that the volume density of adipose cells as well as connective tissue proper cells were lower (P<0.01) in groups without EWF treatment compared to other groups.

DISCUSSION

Between the groups subjected to EWF treatment, the groups fed the CoQ_{10} supplemented diet had a lower volume density of necrotic muscle cells, as well as a higher volume density of non-necrotic muscle cells. The cause of this could be related to the antioxidant properties of CoQ_{10}. Excessive wing flapping is known to lead to ischemia and hypoxia [25].

Under hypoxic conditions, oxidative stress may be induced [14], which results in cell necrosis, tissue damage, and structural modifications within skeletal muscles [12,26]. These effects of oxidative stress can be reduced by anti-oxidants; so CoQ_{10} could decrease changes in tissue structure and cell necrosis and be responsible for the lower volume density of necrotic muscle cells in broilers fed the CoQ_{10} supplemented diet.

Fig 1. Macroscopic (left column) and microscopic (right column) characteristics of deep pectoral myopathy (DPM) stages in broiler chickens. A: No signs of DPM (DPM 0); B: no changes in the structure of deep pectoral muscle (DPM 0), H&E, Bar = 50 µm; C: red-colored areas in deep pectoral muscle (DPM 1); D: numerous leucocytes in damaged parts of muscle (DPM 1), H&E, Bar = 50 µm; E: pale plum colored muscle with a fibrotic structure (DPM 2); F: necrotic muscle cells *(arrows)* in deep pectoral muscle (DPM 2), H&E, Bar = 50 µm; G: greening in central parts of muscle (DMP 3); H: fibrous tissue surrounds necrotic muscle cells (DPM 3), Mallory trichrome, Bar = 50 µm; I: final stage of myopathy (DPM 4); J: replacement of damaged muscle cells *(arrows)* with fibrous tissue (DPM 4), Mallory trichrome, Bar = 50 µm. DP: deep pectoral muscle, SP: superficial pectoral muscle

The use of different classifications used by authors in determining the stage of DPM could lead to misunderstandings. In papers so far, 3-stage [3] and 4-stage [8] classifications can be found. In order to classify DPM stages more precisely, both macroscopically and microscopically, we applied the 4-stage classification as used by Kijowski and Kupińska [8].

In previous studies in which broilers were subjected to forced wing flapping [8] or EWF [9] at 5 days before slaughter (similar to the current experiment) results obtained were in agreement with ours. Similary, Kijowski and Kupińska [8] also observed all 4 stages of DPM in Ross 308 and Flex line broilers that were subjected to forced wing flapping. In male broilers (Ross x Cobb 500) subjected to EWF, a 71% prevalence of DPM lesions was detected by Lien et al.[9], which supports the current findings in the group fed a basal diet and subjected to EWF.

If we surmise that the antioxidant CoQ_{10} can reduce muscle tissue damage and cell necrosis caused by oxidative stress [10,15], it can be assumed that a consequence of that reduction could be a lower average DPM stage in groups of broilers fed a CoQ_{10} supplemented diet and treated with EWF, as compared to groups of broilers fed a basal diet and treated with EWF. This points out the direct relationship between DPM stage and the degree of tissue damage and cell necrosis. Our results were in agreement with the above mentioned theories; between the groups that were subjected to EWF, the volume density of necrotic muscle cells was lower in groups fed the CoQ_{10} supplemented diet.

Examination of DPM in house reared broilers [27] report similar observations as were noticed in our study, such as greenish, pale, and swollen muscles with the presence of necrotic and hemorrhagic tissue that were visible during dissection of the pectoral muscle. In addition, leucocyte infiltration, necrotic muscle fibers, large necrotic areas, and fibro-adipose tissue were detected during microscopic examinations.

The question arises, if the EWF treatment, which in our study lasted 45 seconds, is prolonged, how would this be reflected in the signs of DPM. Kijowski and Kupińska [8] show that increased duration of wing activity leads to greater fatigue of the pectoral muscle, which results in a reduced number of wing flaps in additional time. Therefore, it can be surmised that prolonged duration of EWF treatment will lead to enhanced signs of DPM, but not in proportion to the extended time.

Determination of the most suitable day of age of broilers for the application of EWF treatment is very important. In our study, broilers were subjected to EWF treatment at the end of their 37th day, based on previous findings [8,9] where treating broilers too early in their life resulted in almost complete absence of DPM; this was because of the low weight of the pectoral muscle in which symptoms of myopathy were unable to develop. Meanwhile the application of treatment a short time before slaughter, did not allow time for the later stages of DPM to develop.

Fathi [17] showed that differences in total mortality percentage of broilers due to ascites existed between 2 groups fed diets supplemented with 20 mg and 40 mg of CoQ_{10} per kg of feed. In previous studies, the age at which broilers were offered CoQ_{10} supplements differed. Huang et al.[28] offered broilers a CoQ_{10} supplement diet from 1 day post-hatching, while Fathi [17] offered CoQ_{10} supplement at day 15. As in our experiment, broilers were treated with 20 mg of CoQ_{10}/kg of finisher diet from days 36 to 42; in further research it would be useful to apply different levels of CoQ_{10} in treatments and/or to apply the supplemented diets to broilers of different ages.

We were mindful of animal welfare when selecting EWF in this study as the means for inducing DPM. In previous studies [29-31], the applied methods, such as surgical occlusion of the vascular supply, electrically-induced contractions of the muscle, and forced wing exercise, were more stressful and/or painful to broilers than EWF.

Intensive wing flapping is the main reason for the occurrence of DPM in broilers, and by reducing it the incidence of DPM could be decreased. In order to successfully reduce signs of DPM in broilers, several measures must be applied such as reduced human activity in broiler houses as well as animal activity around the house, refraining from catching birds by their wings, providing sufficient space for every bird during resting, moving, eating, or drinking, etc. These measures are in line with the flock management guidelines presented by Bilgili and Hess [3] in order to reduce the incidence of green muscle disease in broiler flocks.

In conclusion, results indicate that, in broilers fed supplemental CoQ_{10}, the average DPM stage as well as effects of DPM on histological parameters of deep pectoral muscle were reduced at 42 days post-hatching. These findings suggest that the antioxidant properties of CoQ_{10} could reduce the effects of DPM on cell necrosis and change in tissue structure, considering that between the groups subjected to EWF treatment, a lower average DPM stage and volume density of necrotic muscle cells, as well as higher volume density of non-necrotic muscle cells were detected in broilers fed supplemental CoQ_{10}.

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