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Effects of dietary CoQ_{10} and α-lipoic acid on CoQ_{10} levels in plasma and tissues of eggs laying hens

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ABSTRACT

In this paper we described the effect of administrated CoQ_{10}, and alfa-lipoic acid on the concentration of total CoQ_{10} in plasma end body tissues of eggs laying hens. Organisms raise a complex network of enzymes, metabolites and molecules with antioxidant activities in order to prevent oxidative damage of theirs bodies. Adequate blood concentrations of small weight molecules ingested with food and food additives are important for the proper functioning of the antioxidant defense. To test this hypothesis we prepared following experiment. Forty weeks old hens were selected from two genotypes; Ross 308 broiler mothers and Lohmann breed hens. Animals were fed for a period of 84 days. Concentrations of supplemented CoQ_{10} and ALA were calculated from feed instruction tables so each hen received an average of approximately 5 mg of CoQ_{10} and 50 mg of ALA per kg of animal weight per day. During the experiment blood samples were taken and at the end of the experiment different body tissues (heart, liver, breast, legs) were collected and analyzed with originally developed HPLC-MS/MS method based selective ionization with LiCl on MRM scanning. We found a number of interesting and unexpected results. Supplemented CoQ_{10} increased concentrations of coenzyme CoQ_{10} in plasma and different hen's tissues. Increased concentration of CoQ_{10} is the result of its transfer with chylomicrons from the digestive tract to various organs of the body and to the liver where exogenous and endogenous CoQ_{10} has been re-redistributed through lipoproteins. Supplemented ALA caused much greater concentration of CoQ_{10} in different tissues and plasma then CoQ_{10}. Plausible explanation of our results is such that ALA may regenerates the antioxidants and accelerate the formation of endogenous CoQ_{10} which is distributed with lipoprotein carriers and increases overall concentration of CoQ_{10}. Our experiments definitely show that Lipoic acid beside glutathione promotes also a synthesis of CoQ_{10} and increases the total concentration especially in liver and heart tissues.

Keywords: Laying Hens; Coenzyme Q_{10}; α-Lipoic Acid; Antioxidant Network; Fodder Additive

1. INTRODUCTION

Living organisms have to raise a complex system of enzymes, metabolites and molecules with antioxidant activities in order to prevent oxidative damage of theirs bodies [1,2]. Until recently, scientists believed that each antioxidant worked separately, independently of the others. Research performed at the Packer Lab at the University of California at Berkeley showed that there is a dynamic connection among certain key antioxidants. These special antioxidants operate together and represent a dynamic defense of an organism. Antioxidants in this network terminate oxidation processes by removing or quenching free radicals and are capable of slowing or preventing the oxidation [3].

The expression antioxidant network was first presented by Packer [4], who stated that antioxidants do not act alone but are linked together into a network. Interaction of antioxidants had been already noticed before Packer, but he was the first who outlined a concept of a network based on the five molecules; CoQ_{10}, ascorbic acid (vitamin C), tocopherol (vitamin E), glutathione and lipoic acid. The diagram of the antioxidant network built from reduced and oxidized forms of: lipoic acid, glutathione, CoQ_{10}, vitamin C and vitamin E is presented in **Figure 1**. On the top at standard redox potential of less than –0.315 V is the net supplied with protons from NADH (the reduced form of Nicotinamide adenine dinucleotide NAD^+ a coenzyme found in all living cells), and NADPH (the reduced form of Nicotinamide adenine dinucleotide phosphate $NADP^+$). At –0.220 V $FADH_2$ (the reduced form of a redox cofactor flavin adenine dinucleotide FAD involved in several important reactions in metabolism) supports reduced form of CoQ_{10}. In hydrophilic phases a considerable protection is produced from degradation product with antioxidant activity, like uric

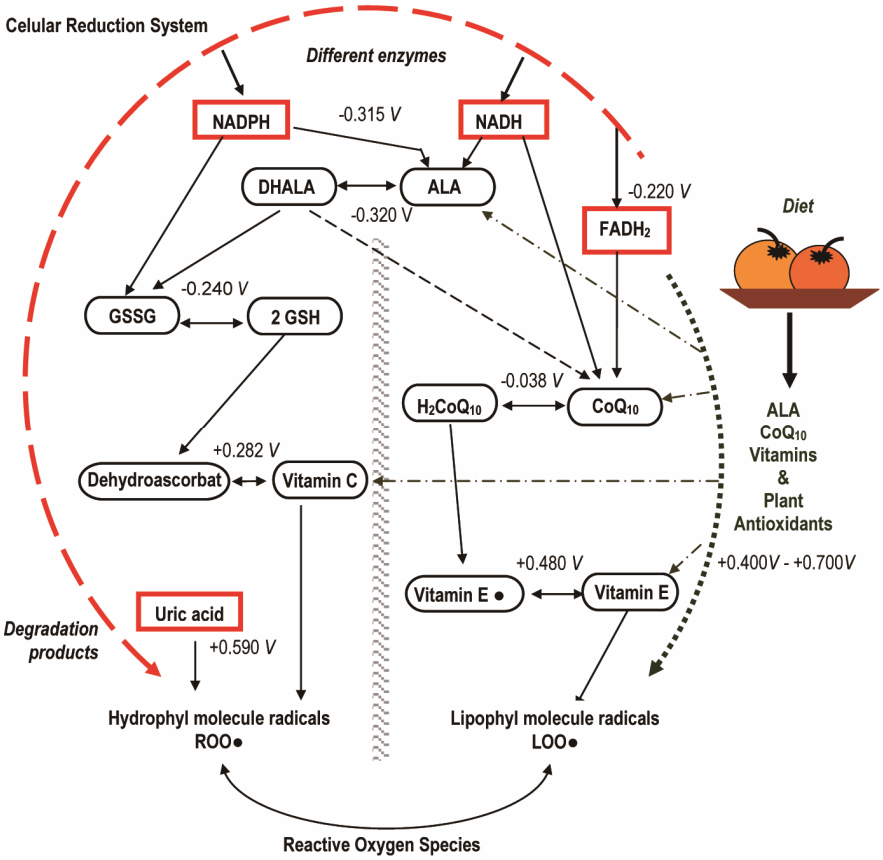

Figure 1. The diagram of the antioxidant network built from reduced and oxidized forms of: lipoic acid, glutation, CoQ_{10}, vitamin C and vitamin E is presented. The net is embedded between the endogenous cellular reduction system and exogenous antioxidants from a diet.

acid at +0.590 V.

Administered food may increase concentrations of vitamins and coenzymes in the network. The antioxidants from plants; carotenoids, flavonoids and polyphenols also protect antioxidant network but only in the redox range between +0.400 V and +0.700 V. From the **Figure 1**, it is possible to conclude that the operation of antioxidant network is complex function [5-7], but also very logical. Regeneration of net strongly depends on high concentration of NADH and NADPH.

Our previous research work connected with industrial poultry farming indicates the hypothesis that chickens and hens could be very suitable candidates for scientific estimation of the intensity of oxidative stress and protective effect of Low Molecular Weight Antioxidants [8,9]. The aim of this study was to determine the effects of a scientifically selected diet on the content of several antioxidants in blood plasma and some animal tissues. Administered food provided necessary conditions for existence of adequate blood levels of enzymes, coenzymes, which together with the large number of administered small weight molecules were responsible for correct functioning of body antioxidant defense. To test this hypothesis we had to develop new reliable analytical methods for assessing the amount of antioxidants in plasma and animal tissues. In present study the concentrations of total amount of CoQ_{10} in different body tissues and blood plasma of laying hens are presented. Oxidized form of CoQ_{10} after prolonged feeding with food fortified with CoQ_{10}, and α-Lipoic acid (ALA) were measured with originally developed HPLC-MS/MS method [10].

2. MATERIALS AND METHODS

2.1. Experimental Design

Forty weeks old hens were selected from two genotypes; Ross 308 broiler mothers and Lohmann breed hens. Animals were housed in wire laying cage (one bird per cage) and fed on the commercial feed NS-val (Ross) and NSK (Lohmann) prepared in Perutnina Ptuj, Slovenia) for 2 weeks before the experiment started. Animals received the supplemented diet on the first day of the experiment and were fed for a period of 12 weeks. Concentrations of supplemented CoQ_{10} and ALA were calculated from feed instruction tables [11,12]. Each hen received an average of approximately 5 mg of CoQ_{10} and (or) 50 mg of ALA

per kg of animal weight per day. During the 84 days pilot raise, all animals were treated under identical environmental and growing conditions. Tests were done in optimal breeding and healthy conditions. The required amount of CoQ_{10} was provided as the water soluble additive originally synthesized in our laboratory (Laboratory for Food Chemistry, National Institute of Chemistry, Ljubljana, Slovenia) by in-capsulation of CoQ_{10} into corn dextrin. The applied food grade alfa-lipoic acid and raw CoQ_{10} were purchased from Linyi Tianliheng Trade Co (China).

During the experiment the blood samples were taken five times, at the start (day 1) and 21, 42, 63, and 84 days after the experiment was introduced. Up to 2 ml of blood were taken from *vene cutaneae ulnaris.* After the end of the experiment hens were sacrificed and different body tissues (heart, liver, breast, leg) were separated and stored together with plasma end blood samples in cool storage at −80˚C until the start of analyses.

All experimental procedures were done according to the guidelines for the care and use of experimental animals at Biotechnical faculty, Department of Animal Science, University of Ljubljana, Slovenia. Experiments on animals were approved by Ethic Committee of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia.

2.2. Materials and Methods

2.2.1. Chemicals

Methanol, ethanol, 2-propanol, 1,4-dioxane, acetonitrile, hexane, perchloric acid and acetic acid (LC grade) were supplied by Merck (Darmstadt, Germany). CoQ_{10} standard and Sodium borohydride were purchased from Sigma Aldrich (Steinheim, Germany). β-Cyclodextrin (food grade) was supplied by Xi'an Hong Chang Pharmaceuticals Co. (China), and CoQ_{10} (pharmaceuticals grade) by Linyi Tianliheng Trade Co (China).

2.2.2. Experimental Procedures

Samples were prepared with following procedures: 400 µL of heparined blood was denaturated with 200 µL of 10% perchloric acid in ethanol. Analites were extracted three times with 2 mL of n-hexane and the combined organic extracts were concentrated with rotary evaporator (Rotavapor R-144 Büchi, Switzerland). The residue was dissolved again in 200 µL of 2-propanol and analyzed with HPLC-ESI-MS/MS.

Part of chicken breasts, legs, wings, whole hearts and livers were mixed with H_2O and homogenized for 3 minutes with Ultraturax at 20.000 rpm into a homogenous paste. 10 g of the homogenized sample were weighed into 50 mL centrifuge tube. 15 mL of warm (35˚C - 40 ˚C) distilled water was added and intensively mixed for 5 minutes. Fat was extracted twice with 20 mL of solvent mixture consisting of chloroform and methanol (2:1, v/v). The combined extracts were concentrated and dried in a stream of nitrogen. The oil residue was dissolved again in 5 mL of 2-propanol.

Plasma and tissues concentrations of total CoQ_{10} were quantified with Sciex API-4000 QTRAP LC/MS/MS system from Applied Biosystems /MDS (Sciex Concord, ON, Canada), equipped with TurboIonSpray^{TM} ionization system and connected to HPLC system constructed from LDC Constametric 4100 pump, and SpectraSystem AS3000 autosampler.

The reduced and oxidized form of CoQ_{10} were successfully separated by LC column-LUNA C18 (2), 3 µm, 100 × 4.6 mm (Phenomenex, Torrance, CA, USA). Both forms were eluted with an isocratic mobile phase (acetonitrile: 2-propanol, 55:45) at a flow rate of 0.5 mL/min. The injection volume was 2.0 µL. For efficient ionization a solution of 0.5 µM LiCl (0.5 mL LiCl/L mobile phase) was added directly into container of mobile phase. Sciex Analyst software was used to perform data analysis and peak integration.

2.2.3. Statistical Analysis

All statistics were run using Statgraphic plus Ver. 4. An analysis of variance (ANOVA) and a Student t-test were employed to evaluate differences between groups with respect to plasma levels, and the relationship between concentration levels and supplementation time.

3. RESULTS

Reliable quantitative determination of CoQ_{10} in biological samples presented in **Tables 1** and **2** was enabled with HPLC-MS/MS analytical method based on improved selective ionization of reduced and oxidized form of CoQ_{10} with added LiCl, and scanning in MRM scan mode. A quasi-molecular ion was formed with the added lithium ion in positive ESI-MS ionization mode. The parent ion for CoQ_{10} was 869.7 m/z $(M + Li)^+$ and selected fragment ion was 241.1 m/z. The linearity rang, was from 0.02 to 5.0 mg/L (ppm), LOD was lower than 0.02 mg/kg and LOQ was 0.04 mg/kg. Obtained sensitivity is nearly 50 times higher than the sensitivity of our previously used analytical methods, mostly single step HPLC-MS.

Nevertheless the new analytical method enables simultaneous determination of reduced and oxidized form, we selected sample preparation with an oxidation step and measured the total CoQ_{10}. In this way effects of uncontrolled oxidation were eliminated [13].

The plasma levels of total CoQ_{10} in chicken's and hen's plasma are shown in **Table 1** and **Figure 2**. Some results are taken from one of our previous study with

Table 1. CoQ_{10} content (mg/L) in the hens and chickens plasma samples. (a) Hens (genotype Ross) started to administer 5 mg CoQ_{10} or 50 mg lipoic acid in 37th week on day 266 and experiment was stopped after 50 weeks on day 350; (b) Laying hens (genotype Lohmann) started with fortified feed, (5 mg CoQ_{10} or 50 mg lipoic acid) on 35th week and experiment was stopped in 47th week; (c) CoQ_{10} content (mg/L) in the chicken plasma (genotype Ross) after daily intake of CoQ_{10} (5 mg) and lipoic acid (50 mg) on kg of body weight. Chickens started with fortified feed on day 16 and experiment was stopped on day 41.

(a)

Day of sampling	CoQ_{10} (mg/L) $\bar{x} \pm sd$		
	$G_{Control}$	$G_{CoQ_{10}}$	G_{ALA}
266	1.99 ± 0.46	2.13 ± 0.79	2.18 ± 0.64
287	1.82 ± 0.51	2.23 ± 0.91	2.78 ± 0.35
308	2.11 ± 0.63	2.50 ± 0.53	3.01 ± 0.75
329	1.77 ± 0.46	2.37 ± 0.42	2.86 ± 0.54
350	1.98 ± 0.38	2.38 ± 0.53	2.90 ± 0.86

(b)

Day of sampling	CoQ_{10} (mg/L) $\bar{x} \pm sd$		
	$G_{Control}$	$G_{CoQ_{10}}$	G_{ALA}
266	2.05 ± 0.80	1.98 ± 0.42	2.06 ± 0.34
287	2.10 ± 0.48	2.23 ± 0.68	3.04 ± 0.50
308	2.03 ± 0.49	2.29 ± 0.51	2.92 ± 0.75
329	2.20 ± 0.60	2.30 ± 0.28	2.95 ± 0.97
350	1.93 ± 0.22	2.38 ± 0.43	2.85 ± 0.76

(c)

Day of sampling	CoQ_{10} (mg/L) $\bar{x} \pm sd$		
	$G_{Control}$	$G_{CoQ_{10}}$	G_{ALA}
16	0.46 ± 0.06	0.47 ± 0.09	0.46 ± 0.09
28	0.59 ± 0.15	0.84 ± 0.33	0.94 ± 0.38
40	0.92 ± 0.26	1.78 ± 0.70	1.34 ± 0.13

chickens and demonstrate a constant increase of CoQ_{10} concentration in chicken plasma [9] during the first weeks of chicken's live. In the control group the starting concentration in day 16 was 0.46 mg/L and the final concentration in day 40 was 0.92 mg/L. At the same time concentrations increased from 0.47 mg/L to 1.78 mg/L in the group which administered CoQ_{10}, and in the group fed with ALA supplement, from 0.46 mg/L to 1.35 mg/L. A similar trend was observed in the experiment with hens. In the control group the level of CoQ_{10} was practically constant, and the average plasma concentration was around 2.0 mg/L. In the test group administering CoQ_{10} the level slightly increased, and the average concentration was about 2.32 mg/L, at the same time in the ALA administering group the average concentration was even higher, 2.75 mg/L. The increased plasma level in animals after administering of CoQ_{10} was seen in many experiments and was expected [14]. Meanwhile the high increase of CoQ_{10} concentration in plasma after ALA supplementation was something new that we did not expect, because so far in the literature was not possible to find such information.

After many repeated experiments we have come to believe that the results obtained are credible and logical effect of ALA antioxidant protection. The research work of Packer 1995, Han 1997 and Sen 1997 and some others [15-17] showed that Lipoic acid could serve as a proglutathione agent and could enhance the cellular level of glutathione (GSH).

Our experiments show that Lipoic acid increases concentrations of CoQ_{10}. From obtained results it was not possible to conclude if the increased concentrations were the result of boosted production of endogen CoQ_{10} or improved protection of exogenous CoQ_{10}. New updated experiments will be needed if we want to clarify the obtained results.

Now our opinion is that both options may be involved, increased production in liver tissue and reduction of oxidative stress which may additionally save the endogen CoQ_{10}. Our experiments have also shown that the increase in CoQ_{10} plasma concentrations in young chickens is greater than in adult hens during the supplementation with CoQ_{10} and ALA. This result may be explained with stronger oxidative stress to which laying hens are exposed.

In **Table 2** are presented concentrations of CoQ_{10} in different tissues of laying hens. In our experiment two genotypes Ross and Lohmann were used. Hans were divided into three groups, control, CoQ_{10}, and ALA group. In each group there were 12 animals of each genotype.

Concentrations of supplemented CoQ_{10} and ALA were calculated and each hen received an average amount of approximately 5 mg of CoQ_{10} or 50 mg of ALA per kg of animal weight, per day. In one group 7 animals of each genotype were selected and followed during the experiment. Plasma, meat and organ samples were taken from the same, at the start selected birds.

Measured values were evaluated in the two different ways. In the first step each genotype was processed separately. In the next step the average values taken from the both genotypes were prepared. These values are shown in **Table 3**.

We selected such solution, nevertheless some significant differences were observed between two genotypes, because we wanted to get enough reliable information related to the difference between supplementation with CoQ_{10} and ALA, regardless of genotype.

Measured values were evaluated in the two different

Table 2. Concentration of CoQ_{10} in different hens tissues after 84 days of supplementation with CoQ_{10} and ALA.

Tissue	Genotype	Control Conc. (mg/kg)	+CoQ_{10} Conc. (mg/kg)	+ALA Conc. (mg/kg)
Liver	Ross	53.7 ± 3.2	48.4 ± 4.5	64.6 ± 9.8
	Lohman	56.1 ± 8.3	59.2 ± 3.6	58.3 ± 14.0
mean		**54.9 ± 1.7**	**53.8 ± 7.6**	**61.5 ± 5.4**
Heart	Ross	55.6 ± 11.6	50.6 ± 10.6	52.4 ± 4.5
	Lohman	51.9 ± 4.2	56.7 ± 10.2	60.2 ± 14.2
mean		**53.8 ± 2.6**	**53.6 ± 4.3**	**57.2 ± 6.8**
Breast	Ross	12.3 ± 1.5	12.3 ± 1.8	14.0 ± 2.7
	Lohman	10.6 ± 0.7	12.1 ± 1.9	12.7 ± 1.3
mean		**11.4 ± 1.2**	**12.2 ± 1.2**	**13.4 ± 0.9**
Leg	Ross	17.2 ± 1.3	18.1 ± 4.7	19.1 ± 4.4
	Lohman	23.5 ± 3.1	26.6 ± 1.7	28.4 ± 0.7
mean		**20.4 ± 4.4**	**22.4 ± 6.0**	**23.8 ± 6.5**
plasma	Ross	1,87 ± 0.42	2.37 ± 0.47	2.75 ± 0.69
	Lohman	2.06 ± 0.41	2.22 ± 0.34	2.76 ± 0.87
mean		**1.97 ± 0.18**	**2.30 ± 0.09**	**2.76 ± 0.04**

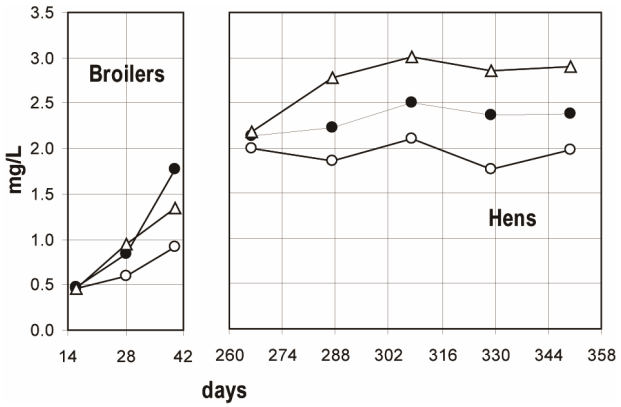

Figure 2. CoQ_{10} content (mg/L) in the plasma of broilers and hens genotype Ross, results are taken from two consecutive experiments. Animals were fed with different fodder additives. Applied labels: (—○—) control group; (—●—) 5 mg CoQ_{10}; (—Δ—) 50 mg ALA on 1 kg of birds weight approximately.

ways. In the first step each genotype was processed separately. In the next step the average values taken from the both genotypes were prepared. These values are shown in **Figure 3**. We selected such solution, nevertheless some significant differences were observed between two genotypes, because we wanted to get enough reliable information related to the difference between supplementation with CoQ_{10} and ALA, regardless of genotype. Calculated values represent the amount of CoQ_{10} in control group and two experimental groups. They were obtained from measured concentrations (mg/kg) multiplied with estimated weights from instruction tables (kg) of processed organs and meat tissues.

In our experiments supplemented CoQ_{10} was not accumulated in liver and heart, but in legs and breasts. An increase of nearly 10% was recorded. The highest increase was seen in plasma, nearly 15%. In the test group which administered ALA the increase of CoQ_{10} was much higher than in coenzyme group. In heart tissue the final level of CoQ_{10} was more than 5% and in liver more than 10% higher than in the control group. Concentrations in meat tissues were very high, more than 15% and in plasma nearly 40% higher than in control group. The same trend is seen in both genotypes groups. Lipoic acid produces much higher concentration of CoQ_{10} then supplemented CoQ_{10} alone. It is interesting that Lohmann hens have much higher response with both supplements. It is also unexpected that concentrations in heart and liver are not increased, in reality in some cases they are even reduced. We explain these results with the influence of oxidative stress which is obviously higher in the Ross group then in the Lohmann group. Our results also show that higher concentration of CoQ_{10} in plasma does not automatically mean high concentrations of CoQ_{10} in tissues.

We tried to clarify the link between distribution, accumulation, and elimination of exogenous and endogenous CoQ_{10} in animal tissues with a help of a model. We wanted to determine if eaten lipoic acid busted a production of new CoQ_{10} or only eliminate oxidation of it. Concentrations of processed tissues were taken from our experiments. The exogenous CoQ_{10} was transported from column to liver with chylomicrons where it was prepacked to Apoproteins and redistributed through the body. In both transport paths, lipoic acid may prevented the decomposition of coenzyme. It also restored certain liver functions [18,19] and in this way boosted the synthesis, which increased the overall concentration of CoQ_{10}. In

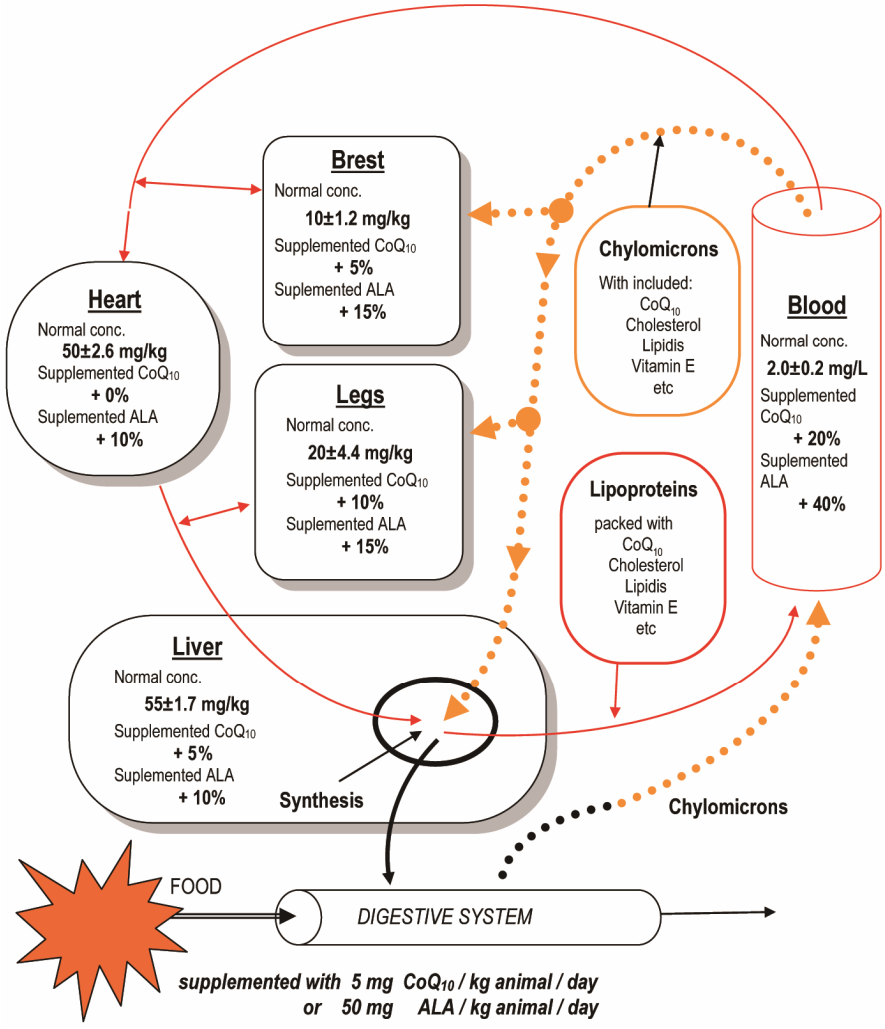

Figure 3. Average CoQ_{10} levels in tissues and plasma of laying hens in control group and after oral administration of CoQ_{10} and ALA are shown. Increased concentrations of CoQ_{10} are expressed in percent.

Table 3. Calculated values (mg/unit) of CoQ_{10} in different organs and body parts of hens of Ross and Lohmann genotype are shown. Measured concentrations of CoQ_{10} (mg/kg) were multiplied with estimated weight of selected body parts.

Samples		Control group		Supplemented CoQ_{10}			Supplemented ALA		
	Weight (g)	*CoQ_{10} mg/kg	CoQ_{10} mg	*CoQ_{10} mg/kg	CoQ_{10} mg	Diff. %	*CoQ_{10} mg/kg	CoQ_{10} mg	Diff. %
Legs R	750	17.2	12.91	18.1	13.59	105.3	19.2	14.37	111.3
Breast R	550	12.3	6.74	12.3	6.77	100.5	14.0	7.70	114.2
Liver R	50	53.6	2.68	48.4	2.42	90.3	64.6	3.23	120.5
Hearth R	25	55.6	2.78	50.6	2.53	90.9	52.4	2.62	94.2
Blood R	250	1.9	0.97	2.3	1.16	120.2	2.75	1.37	142.1
∑ Ross	1625		26.08		26.48	**101.5**		29.30	**112.3**
Legs L	375	23.5	8.81	26.6	9.98	113.3	28.4	10.66	120.9
Breast L	330	10.6	3.49	12.0	3.97	113.9	12.7	4.19	120.0
Liver L	30	56.1	1.68	59.2	1.78	105.5	58.3	1.75	103.9
Hearth L	15	51.9	1.17	56.7	1.28	109.2	62.0	1.40	119.4
Blood L	150	2.1	0.46	2.3	0.51	109.3	2.76	0.62	134.1
∑ Lohmann	900		15.62		17.52	**112.2**		18.61	**119.2**

cells Lipoic acid took care of antioxidant network and protected lipid membranes by elimination of uncontrolled oxidation which resulted in higher levels of CoQ_{10}.

Nevertheless the correlations between measured and calculated values were good, we were not able to conclude which previously described option was prevalent, and further experiments are necessary.

4. CONCLUSION

Lipoic acid is the most potent member of antioxidant protection in a body. With electric potential of (−320 mV) it may regenerate all other antioxidants. Results undoubtedly confirm the existence of an antioxidant network and synergistic effect of administered low weight substances. Our work demonstrates that ALA is able to influence not only on the regeneration of glutathione but according to our results also on regeneration of CoQ_{10}.

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