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EFFECT OF SUPPLEMENTATION WITH METHIONINE AND DIFFERENT FAT SOURCES ON THE GLUTATHIONE REDOX SYSTEM OF GROWING CHICKENS

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The effect of supplementary methionine and fats of different saturation levels on the glutathione redox system of growing broiler cockerels was studied. The diet of three groups of chicks was supplemented with corn germ oil, beef tallow and fish oil at the levels of 30 g/kg and 50 g/kg of feed, respectively. The diet of further three groups was supplemented with methionine (5 g/kg of feed) in addition to the different fat sources. Control chicks were fed with a compound feed without methionine and fat supplementation. Reduced glutathione (GSH) and glutathione disulphide (GSSG) content as well as glutathione peroxidase activity in the liver were determined and GSH/GSSG ratio was calculated at day old and then at one and three weeks of age. Our results indicate that supplementary methionine stimulates both the synthesis of the glutathione redox system and glutathione peroxidase activity in growing chickens in the first period of postnatal life, when the risk of lipid peroxidation is high due to feeding unsaturated fats in the diet.

Key words: Methionine, fats, lipid peroxidation, glutathione redox system, growing chickens

Numerous endogenous antioxidants provide protection against cellular oxidation, oxygen and other derived free radicals, toxins and other mediators of oxidative stress (Frei, 1994). Of them, glutathione (GSH) is a thiol antioxidant ubiquitous in cells, which has a fundamental role in the detoxification of xenobiotics and free radicals, and serves also as a cysteine store and as a xenobiotic-conjugating compound (DeLeve and Kaplowitz, 1991). Glutathione (GSH) is synthesised from its constituent amino acids mainly in the liver and transported

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to different tissues by the systemic circulation (Wang et al., 1997). Its antioxidant effect has been confirmed by the results of studies in which a rapid and substantial decline of glutathione level was observed as a result of oxidative stress (Daba and Abdel Rahman, 1998). The synthesis of GSH is inhibited by the formed peptide through negative feedback, and it is affected also by the actual methionine and cysteine supply (Wang et al., 1997). Methionine has an important role in the glutathione redox system because it can be converted into cysteine, a component of glutathione (Singer, 1975) through cystathionine by transsulphuration (Greenberg, 1975).

Among the components of the glutathione system, reduced glutathione (GSH) is one of the most sensitive indicators of oxidative stress (Troncoso et al., 1997). Supplementation of the feed with fish oil rich in n-3 polyunsaturated fatty acids (PUFA) is characterised by a decline of GSH, in response to the increase of lipid peroxidation in the tissues (D'Aquino et al., 1991). The oxidised component of the glutathione redox system, glutathione disulphide (GSSG) indicates oxidative stress in the same way as GSH. In addition to changes of the level of GSH and GSSG, the GSH/GSSG ratio is a more accurate and sensitive indicator of oxidative stress (Storey, 1996). The glutathione peroxidase (GSHPx) enzyme family is an important representative of the enzymatic biological antioxidant system. The function of the enzyme is to reduce hydrogen peroxide and organic peroxides in the presence of reduced glutathione which also acts as free radical scavenger, while GSH is oxidised (Hornsby and Crivello, 1983). Classical or cytoplasmic GSHPx is ubiquitous in tissues characterised by marked peroxide production, such as kidney, liver and lungs (Chambers and Harrison, 1988). In experiments with rats, the activity of GSHPx was found to be increased in the liver when the proportion of PUFA was higher in the diet (D'Aquino et al., 1991).

The objective of the present experiment was to study the effect of supplementing the diet with methionine and fats containing fatty acids of different saturation level on the glutathione redox system in broiler cockerels in the first 3 weeks of postnatal development. Evaluation of the amount and activity of the glutathione system was based on the levels of GSH and GSSG as well as on the activity of GSHPx in the liver. The GSH/GSSG ratio was calculated from the values measured.

Materials and methods

Experimental animals and diets

Cockerel chicks of Ross 308 genotype were reared in battery cages (50 chicks per treatment) from day old to 3 weeks of age. The chicks were fed on a starter diet up to 14 days of age and then on a grower diet up to the end of the experiment. A basic compound feed without fat and methionine supplementation

(control) was prepared for the experiment (Table 1). Vitamins, minerals and essential amino acids were in quantities conforming to the recommendations of the provided Hungarian Feed Codex (1990). The diet of three experimental groups was supplemented with beef tallow (Zalahús Co. Ltd., Zalaegerszeg) rich in saturated and monounsaturated fatty acids, with corn germ oil (Corn Drop Ltd., Szabadegyháza) rich in n-6 fatty acids and with fish oil (Helikon Pharmacy, Keszthely) rich in n-3 fatty acids, respectively. The level of fat supplementation of the experimental diets was 30 g/kg and 50 g/kg of feed in the starter and grower phase, respectively. The diet of further three experimental groups was supplemented with methionine (5 g/kg, Europharma Ltd., Budapest) in addition to different fat sources. Feed and drinking water were provided *ad libitum*.

The experiments were authorised by the Scientific Ethics Committee for Animal Experiments of the Advisory Council on Animal Experiments (permit number: 116/2001.03.28).

Table 1

Composition and nutrient content of the control diet

Ingredients	Quantity (g/kg)	
	Starter	Grower
Wheat	150.00	150.00
Maize	488.00	530.00
Soybean meal (46%)	306.00	280.00
Fishmeal (64%)	20.00	10.00
MCP	9.00	5.00
Fodder lime	2.00	–
Vitamin and mineral premix*	25.00	25.00
Total	1000.00	1000.00
Calculated nutrient contents		
$ME_{poultry}$ (MJ/kg)	12.59	12.82
Crude protein (g/kg)	194.8	180.7
Methionine (g/kg)	5.0	4.9

*Provides per kilogram of vitamin/mineral premix: 65 g/kg methionine; 300,000 IU vitamin A; 84,000 IU vitamin D_3; 600 mg vitamin E; 75 mg vitamin K_3; 60 mg vitamin B_1; 162 mg vitamin B_2; 48 mg vitamin B_6; 0.48 mg vitamin B_{12}; 14,520 mg choline chloride; 30 mg folic acid; 300 mg pantothenic acid; 900 mg nicotinic acid, 1956.77 mg Zn; 1251.17 mg Fe; 5.34 mg Co; 712.17 mg Cu; 1674.46 mg Mn; 20.32 mg I; 7.42 mg Se; 200.00 mg maduramicin

Sample collection

Liver samples were taken at day old and then at 1 and 3 weeks of age. The chickens were slaughtered by decapitation and the liver was removed immediately after exsanguination. At each sampling, chickens were selected randomly in a number corresponding to five replications. The livers were pooled before the analysis: one sample consisted of livers of five chicks at day old, three chicks at one week of age and two chicks at 3 weeks of age. The samples were homogenised with a knife blender. Subsequently 1 g of the sample was suspended with 9 ml of 0.9% NaCl solution at +4 °C in a Potter-Elvehjem apparatus, then centrifuged at 15,000 rpm at +4 °C for 15 min. The supernatant fraction was frozen immediately after separation and stored at –20 °C until analyses.

Table 2

Fatty acid composition of supplementary fats used in the experiments

Fatty acids	Corn germ oil	Beef tallow	Fish oil
	percentage of total fatty acids		
C14:0	–	3.5	7.5
C16:0	10.3	28.3	13.8
C16:1 n-7	0.1	7.8	13.3
C18:0	2.0	10.7	2.0
C18:1 n-9	30.7	46.7	24.7
C18:2 n-6	54.3	1.0	1.9
C18:3 n-3	1.0	–	8.1
C20:1 n-9	–	–	4.1
C20:4 n-6	–	–	–
C20:5 n-3	–	–	9.1
C22:4 n-6	–	–	0.3
C22:5 n-3	–	–	1.4
C22:6 n-3	–	–	8.8
Others	1.6	2.0	1.9
SAT[1]	12.3	42.5	23.3
MUFA[2]	30.8	54.5	42.1
Total n-3	1.0	–	27.4
Total n-6	54.3	1.0	6.3
PUFA[3]	55.3	1.0	33.7

[1]SAT = saturated fatty acids; [2]MUFA = monounsaturated fatty acids; [3]PUFA = polyunsaturated fatty acids

Chemical analyses

Reduced glutathione content of the liver homogenate 10,000 *g* supernatant fraction was measured by the method of Sedlak and Lindsay (1968). The quantity of glutathione disulphide was determined according to Tietze (1969), while glutathione peroxidase (E.C. 1.11.1.9.) activity by the method of Lawrence and

Burk (1978). Enzyme activity was expressed in units (U): one unit means the oxidation of 1 nmol glutathione per minute at 25 °C. The activity of the enzyme was related to the protein content of the 10,000 *g* supernatant fraction of the liver homogenate, which was determined using Folin phenol reagent (Lowry et al., 1951). The fatty acid composition of the different fat sources (Table 2) was determined as described by Husvéth et al. (1982).

Statistical analysis

The results were evaluated by analysis of variance (ANOVA) using SAS (1990) software. Where the *F* test indicated statistically significant difference, the differences between the individual treatments were compared by the Tukey test (SAS, 1990).

Results

The GSH content (Table 3) of the liver was higher in all groups at one week of age than the value measured in day old chicks (461.1 nmol/g). In addition, higher GSH concentrations were measured in both the methionine-supplemented and the fat-supplemented groups than in the control. Also at three weeks of age, higher GSH content was found in the liver of chicks fed on diets supplemented with both methionine and various fats, respectively or combined, as compared to the control.

The GSSG content of the liver (Table 3) of the one-week-old chicks in the control group was significantly ($P < 0.05$) higher than in the day-old chicks (5.2 nmol/g). At that sampling time, the groups fed on diets containing any fat without methionine supplementation showed lower GSSG content than the control. In the liver of three-week-old chicks the methionine supplementation caused a significant ($P < 0.05$) decrease in the GSSG content.

The GSH/GSSG ratio in the liver (Fig. 1) of one-week-old chicks fed supplementary fats of different saturation levels was significantly ($P < 0.05$) higher compared to the control group. Higher level of methionine in combination with beef tallow or fish oil significantly ($P < 0.05$) increased the GSH/GSSG ratio even when compared to groups fed on diets supplemented with beef tallow and fish oil. At three weeks of age, methionine supplementation resulted in higher GSH/GSSG ratios in the case of all types of fat as compared to the groups without methionine supplementation.

In relation to GSHPx activity measured in day-old birds (1.2 U/g), a significantly lower activity was found at one week of age in chicks fed diets supplemented with fats of different saturation level. At that age, in birds fed with methionine and fat in combination, GSHPx activity (Table 3) was significantly ($P < 0.05$) higher than in the liver of chicks fed only fat-supplemented diets. This tendency was also shown in the liver of three-week-old chicks.

Table 3

Change of parameters of the glutathione redox system in the liver

Experimental diets	1-week-old chickens			3-week-old chickens		
	GSH nmol/g	GSSG nmol/g	GSH-PX U/g	GSH nmol/g	GSSG nmol/g	GSH-PX U/G
C	503.0 ± 19.6^{b}	9.9 ± 1.0^{a}	1.5 ± 0.1^{b}	433.5 ± 28.6^{b}	5.1 ± 0.3^{bc}	1.2 ± 0.1^{a}
ME	573.6 ± 54.1^{b}	10.4 ± 0.5^{a}	2.5 ± 0.3^{a}	564.4 ± 38.7^{ab}	3.4 ± 0.2^{de}	0.7 ± 0.1^{b}
BT	533.7 ± 24.9^{b}	5.7 ± 0.3^{c}	1.0 ± 0.1^{bc}	565.9 ± 18.1^{ab}	6.0 ± 0.2^{ab}	0.5 ± 0.5^{b}
BT-ME	829.6 ± 14.0^{a}	4.8 ± 0.2^{c}	1.6 ± 0.2^{b}	638.1 ± 46.0^{a}	3.4 ± 0.3^{de}	1.1 ± 0.0^{a}
CO	630.7 ± 27.8^{b}	6.3 ± 0.4^{c}	1.0 ± 0.2^{bc}	554.9 ± 47.2^{ab}	6.4 ± 0.4^{a}	0.6 ± 0.1^{b}
CO-ME	681.9 ± 43.6^{ab}	8.7 ± 0.2^{ab}	1.5 ± 0.1^{b}	580.8 ± 37.7^{ab}	4.4 ± 0.1^{cd}	1.4 ± 0.1^{a}
FO	564.0 ± 55.4^{b}	6.4 ± 0.4^{bc}	0.6 ± 0.1^{c}	473.7 ± 4.3^{b}	2.8 ± 0.1^{e}	0.5 ± 0.1^{b}
FO-ME	673.2 ± 48.6^{ab}	4.9 ± 0.4^{c}	3.0 ± 0.1^{a}	554.2 ± 34.9^{ab}	2.4 ± 0.1^{e}	1.2 ± 0.1^{a}
Level of significance						
Fat	*	***	***	*	***	*
Methionine	***	NS	***	**	***	***
Fat × Methionine	*	**	***	NS	***	***

C = control; ME = C + 5 g/kg methionine; CO = C + 30 g/kg or 50 g/kg (starter or grower) corn germ oil; CO – ME = CO + 5 g/kg methionine; BT = C + 30 g/kg or 50 g/kg (starter or grower) beef tallow; BT – ME = BT + 5 g/kg methionine; FO = C + 30 g/kg or 50 g/kg (starter or grower) fish oil; FO – ME = FO + 5 g/kg methionine. The table shows the means ± standard error of the mean (SEM; n = 5) for the different treatments. Different letters (a–e) denote significant ($p < 0.05$) differences between the means in the same column. NS = non-significant; $^{*} = P < 0.05$; $^{**} = P < 0.01$; $^{***} = P < 0.001$

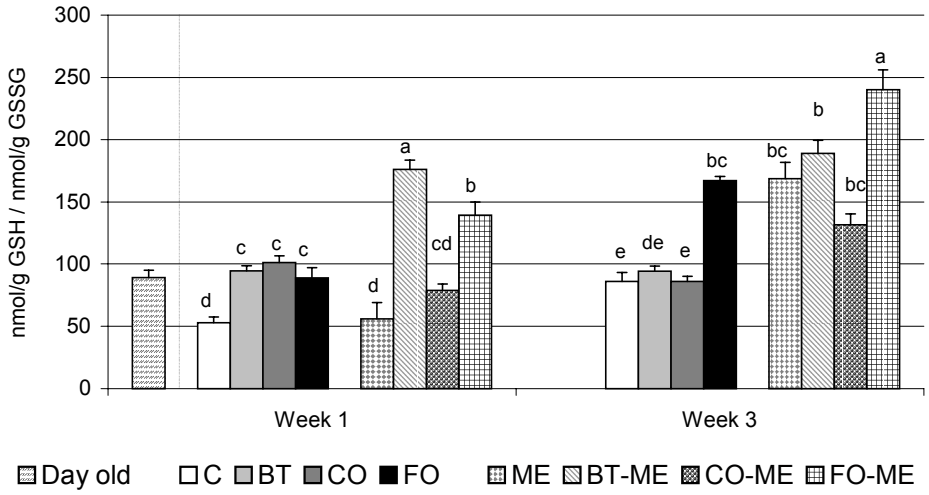

Fig. 1. Change of GSH/GSSG ratio in the liver tissue. The figure shows the means ± standard error of the mean (SEM) for the different treatments. Different letters denote significant ($P < 0.05$) differences between the means at the sampling time. D = day old, i.e. basal value for all groups excluded from the statistics; C = control; ME = C + 5 g/kg methionine; CO = C + 30 g/kg or 50 g/kg (starter or grower) corn germ oil; CO – ME = CO + 5 g/kg methionine; BT = C + 30 g/kg or 50 g/kg (starter or grower) beef tallow; BT – ME = BT + 5 g/kg methionine; FO = C + 30 g/kg or 50 g/kg (starter or grower) fish oil; FO – ME = FO + 5 g/kg methionine

Discussion

As a result of dietary supplementation with energy sources of different fatty acid composition, the concentration of GSH increased at one and three weeks of age, respectively, in the liver of cockerels as compared to the control. These results are partially consistent with some earlier observations (Wang et al., 1997) which showed that the GSH level in the liver of broiler chickens gradually increases with age. Fish oil supplementation increased the GSH content of the liver, which is at variance with an earlier report (D'Aquino et al., 1991) that fish oil of high n-3 fatty acid content diminishes the GSH content in the liver. Although the feeding of diets with high unsaturated fatty acid content is expected to decrease the GSH level, the opposite may occur if the effect of the applied fish oil is not manifested at the applied dose. In both age groups the methionine supplementation increased the concentration of GSH in the liver, which was indicative of enhanced glutathione synthesis. This is based on the well-known fact that both methionine and cysteine are key factors in glutathione synthesis (Singer, 1975).

Fatty acids of different saturation levels show different sensitivity to oxidative damage, which presumably affects the amount and/or activity of the antioxidant, including the glutathione redox system (Mataix et al., 1998). Supple-

mentation of fats with different saturation levels and combination with methionine exerted dissimilar effects on the GSH level, which supports the earlier hypothesis of ourselves and others (D'Aquino et al., 1991; Crosby et al., 1996) concerning the effect of unsaturated fatty acids on the glutathione redox system.

As opposed to the expected oxidative effects, the amount of GSSG decreased, rather than increased, in most cases as a result of fat supplementation. No exact explanation of this finding can be given based on the results of the present study. The possible cause of this result may be that the GSH level increased due to fat and methionine supplementation and thus, the absolute amount of the oxidation products became lower, because of the higher absolute thiol content of the cells. This might result in a higher rate of both GSH and GSSG efflux. The changes of GSH and GSSG content of the liver were irrespective of fatty acid composition because at three weeks of age even the mostly unsaturated corn germ oil or the saturated beef tallow supplementation caused the same increase in the level of GSH as compared to the control diet, while fish oil containing also highly unsaturated fatty acids had no significant effect on GSH. Otherwise, fish oil supplementation showed the highest rate of decrease of GSSG formation compared to the control. These changes were indicative of different effects of fat supplementation on thiol content of the cells.

Irrespective of the treatments, the GSH/GSSG ratios increased in the first three weeks of postnatal life as compared to the control, which suggested the enhancement of glutathione synthesis. The opposite tendency occurred only as a result of corn germ oil supplementation, which indicates that the fatty acid composition of that fat source imposed an increased load on the glutathione redox system. Namely, at identical levels of GSH synthesis the GSH/GSSG ratio will decrease only if enhanced oxidation is taking place simultaneously in the given tissue. The stimulatory effect of methionine supplementation on GSH synthesis manifested throughout the experiment. However, the elevated GSH level did not yet inhibit the intensity of the synthesis through negative feedback (Wang et al., 1997). The continuous increase of the GSH/GSSG also indicates that enhanced oxidation of GSH need not be reckoned with in the case of methionine supplementation, i.e. the antioxidant system provides adequate protection against the increased unsaturated fatty acid load.

As compared to the control group, the activity of glutathione peroxidase decreased as a result of fat supplementation while it was markedly enhanced by methionine supplementation. This activity elevation presumably occurred as a result of increased substrate (i.e. glutathione) supply (Flohé, 1989). However, it should be mentioned that a GSH concentration exceeding the saturation level lowers the enzyme activity (Maddipati and Marnett, 1987). The other possibility is that surplus cysteine formed as a result of methionine supplementation activates the synthesis of selenocysteine, the active site of GSHPx, which may increase the enzyme activity (Chambers et al., 1986). Unsaturated fatty acids gen-

erally increase the activity of GSHPx (Bellisola et al., 1992), and therefore some other factors, which cannot be evaluated accurately from the results of the present experiment, may play a role in the decreased activity of GSHPx. When beef tallow and methionine supplementation was used in combination, GSHPx activity increased whereas corn germ oil or fish oil rich in unsaturated fatty acids lowered it. This latter effect may be based on the effect of hydroperoxides formed from unsaturated fatty acids (Bellisola et al., 1992).

In conclusion, methionine supplementation was found to enhance glutathione synthesis in the liver of cockerels in the first three weeks of postnatal life. The unsaturated fatty acids impose a substantial load on the glutathione redox system. At the same time, different types of unsaturated fatty acids (n-3, n-6) stimulate the glutathione redox system in different ways. This effect may be attributed to the dissimilar sensitivity of n-3 and n-6 fatty acids to oxidation. Determination of the exact mechanism of action requires further investigations. Our results obtained on components of the glutathione redox system indicate the favourable effect of methionine supplementation. The high unsaturated fatty acid content of diets compromises the antioxidant status of broiler chickens. The technological recommendations (National Research Council, 1994; Ross Breeders Ltd., 1999) give the methionine level required for growth in chickens. In order to decrease the risk of lipid peroxidation in chicks fed on a diet containing a higher level of unsaturated fatty acids, further methionine supplementation would be needed for stimulating the synthesis of the antioxidant glutathione.

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