Effect of melamine and cyanurid acid contaminated diets on blood indicators in broiler chickens

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ABSTRACT: The effect of melamine and cyanuric acid contaminated diets on basic haematological and biochemical blood indicators in male broiler chickens (Ross 308) was studied. The chickens were divided into 6 experimental groups (30 birds per group) and fed diets with an addition of 50 or 100 mg of melamine or 50 or 100 mg of cyanuric acid per kg of feed, with the contaminants added separately (either melamine or cyanuric acid) or in combination (melamine + cyanuric acid). The control group (C) was fed a diet without melamine or cyanuric acid. At the end of the experiment (day 40), 8 birds per treatment group were randomly selected for haematological and biochemical examination of blood. Red blood cell count, haemoglobin concentration, haematocrit value, mean corpuscular haemoglobin concentration, mean corpuscular haemoglobin, mean cell volume, and total leukocyte count did not differ significantly among the respective groups of broiler chickens ($P > 0.05$). The concentrations of melamine and cyanuric acid used in the contaminated diet for broiler chickens led to highly significant changes in the content of total protein (TP), glucose (Glu), Ca, P, Na, and K ($P < 0.01$) and to significant changes in the content of Mg ($P < 0.05$) in their blood plasma. In comparison to the C group, the decrease in the content of TP, Glu, Ca, P, Na, K, and Mg in blood plasma was most pronounced in broilers fed the diets contaminated with cyanuric acid only or diets with the simultaneous contamination with melamine and cyanuric acid. On the basis of our results, it can be concluded that the contamination of feed with melamine and cyanuric acid, separately or in combination, results in impaired renal function and probably also in partial liver damage.

Keywords: poultry; feed; level of contaminants; haematological indicators; biochemical indicators; toxicity

List of abbreviations: AST = aspartate aminotransferase, Chol = cholesterol, Er = total red blood cell count, Glu = glucose, Hb = haemoglobin concentration, Ht = haematocrit value, Leu = total leukocyte count, MCH = mean corpuscular haemoglobin, MCHC = mean corpuscular haemoglobin concentration, MCV = mean cell volume, TP = total protein, UA = uric acid

INTRODUCTION

Melamine (2,4,6-triamino-1,3,5-triazine) is an organic compound widely used in industry especially to produce varnishes, paints (Singh and Kumar 2009), plastic packages, and food containers (Lu et al. 2009). Through these products, it may enter the human as well as the animal food chain as an unintended contaminant. It is reported that melamine is relatively non-toxic, however, comprehensive information on its toxicity is still missing (Puschner et al. 2007). Based on the EU regulation, the specific migration limit of melamine content in materials intended to come into contact with food should not exceed 30 mg/kg (Commission Directive 2002/72/EC). According to Lu et al. (2009), the migration of melamine from packaging materials into food is relatively low, however, the

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melamine itself has many analogues – ammeline (4,6-diamino-2-hydroxy-1,3,5-triazine), ammelide (6-amino-2,4-dihydroxy-1,3,5-triazine), and cyanuric acid (2,4,6-trihydroxy-1,3,5-triazine). In combination with cyanuric acid, melamine might form an insoluble compound (melamine–cyanurate complex) which can cause death due to renal failure (Kobayashi et al. 2010).

From chemical point of view, melamine contains high amount of nitrogen (67%), which was misused by some food producers who added melamine to their products to mask the insufficient content of nitrogen-containing substances. There were many cases of renal failure in children in China where melamine was added to infant formula (Cao et al. 2013). Melamine and cyanuric acid were also detected in feed for cats and dogs in the U.S. as a component accompanying wheat gluten, rice protein, and corn gluten imported from China, again probably with the objective to increase the content of nitrogen-containing substances, however, as a result a nephrotoxic renal failure developed in lots of dogs and smaller number of cats (Brown et al. 2007). It was confirmed that the combination of melamine and cyanuric acid is responsible for acute renal failure and the presence of renal crystals not only in dogs and cats (Puschner et al. 2007), but also in pigs and fish (Reimschuessel et al. 2008). Risk of feeding animals a diet contaminated with melamine and cyanurid acid was stated also by Dobson et al. (2008) who detected a forming of an insoluble precipitate in renal tubules leading to progressive tubular blockage and degeneration in rats and cats. The presence of cyanuric acid in contaminated feed can be the result of intentional addition but also the result of unintended contamination caused by the presence of melamine, cyanuric acid being its by-product (Hau et al. 2009).

Hau et al. (2009) stated that melamine is not metabolized in animals and 90% of it is excreted via kidneys during 24 h after consumption. However, Zheng et al. (2013) found that in rats, melamine was biotransformed by intestinal microorganisms (namely by *Klebsiella terrigena*) into cyanuric acid which subsequently became the main component of kidney stones. It can therefore be assumed that similar processes occur in other animal species.

Regarding melamine and its derivates, they can also be stored in the body and when these compounds are present in diets for food animals, they represent risk to human health as well. Suchy et al. (2014) reported that in laying hens not only melamine was stored in their tissues, but it was also biotransformed into cyanuric acid which had not been confirmed in poultry before. The experimental administration of melamine contaminated diets to laying hens also resulted in the excretion of some part of melamine into eggs (Bai et al. 2010; Novak et al. 2012).

Melamine metabolism and its toxicity in poultry have not been fully understood yet. It is possible that exposure limits for feeds which had been established previously may in fact be lower than previously assumed. Therefore the aim of this study was to evaluate the effect of diets contaminated with various concentrations of melamine and cyanuric acid on basic haematological and biochemical indicators in broiler chickens, including the relation to potential damage of internal organs.

MATERIAL AND METHODS

Two hundred and ten male broiler chickens (Ross 308) were used in the experiment. One-day old broiler chickens were randomly placed in 7 groups (1 control and 6 experimental groups; 30 birds per group). Chicks of each group were housed in one-floor pen covered with wood shavings. Broiler chickens were housed in the accredited experimental stable of the Department of Animal Nutrition and the Department of Animal Husbandry and Animal Hygiene, University of Veterinary and Pharmaceutical Sciences in Brno (UVPS Brno) under controlled housing conditions that fully complied with the standards used for fattening of Ross 308 broilers. The experimental procedures were approved by the Animal Welfare Committee of UVPS Brno (1/2011/2220/FVHE).

A two-phase feeding program (starter diet in days 1–15 and finisher diet in days 16–40; small-sized granules) was applied. The ingredient and nutrient composition of the basal diets is outlined in Table 1.

The chickens in the control (C) group were fed basal diets without melamine or cyanuric acid. For the experimental groups, melamine (M2659; Sigma-Aldrich Chimie S.a.r.l., Saint-Quentin Fallavier, France) and cyanuric acid (185809; Sigma-Aldrich Chimie S.a.r.l.) alone and in combination were added to the basal diets. The M50 and M100 groups were given the basal diet with the addition of 50 or 100 mg melamine per kg of feed, respectively; the K50 and K100 groups were given the basal diet with the addition of 50 or 100 mg of cyanuric acid per kg of feed, respectively; the MK50 group was

Table 1. Ingredients and nutrient composition of the basal diets

	Starter diet (days 1–15)	Finisher diet (days 16–40)
Ingredients (%)		
Wheat	41.20	47.73
Soybean meal	35.79	29.60
Maize	15.00	15.00
Soy oil	3.60	3.60
Limestone	1.63	1.63
Monocalcium phosphate	1.18	1.00
NaCl	0.38	0.38
DL-Methionine	0.30	0.25
L-Lysine	0.30	0.23
L-Threonine	0.12	0.08
Premix Mikrop[1]	0.50	0.50
Nutrient composition (g/100g as fed)		
Dry matter	88.23	88.64
Crude protein	21.84	19.85
Crude fat	4.62	6.40
Crude fibre	2.00	2.74
Nitrogen-free extractives	54.37	55.27
Organic matter	82.83	83.90
Crude ash	5.40	4.74
Ca	1.50	0.82
P	0.60	0.51
Mg	0.15	0.26
Metabolizable energy (MJ/kg)	13.30	13.60

[1]contents in 1 kg of Premix: vitamin A 8000 IU, vitamin D_3 2500 IU, alpha-tocopherol 50 mg, vitamin K_3 1.5 mg, vitamin B_1 4 mg, vitamin B_2 6.5 mg, vitamin B_6 3 mg, vitamin B_{12} 0.015 mg, biotin 0.15 mg, folic acid 2.5 mg, nicotinic acid 30 mg, calcium pantothenate 12.5 mg, betaine 250 mg, butylhydroxytoluene 17 mg, propyl gallate 6 mg, ethoxyquin 2.7 mg, ferrous sulphate monohydrate 50 mg, manganese oxide 80 mg, zinc oxide 80 mg, copper sulphate 8.7 mg, potassium iodide 1 mg, sodium selenite 0.15 mg, cobalt sulphate 0.25 mg, phytase 250 FTU, glucanase 120 BGU, xylanase 5500 EXU

given the basal diet supplemented with 50 mg/kg of melamine and 50 mg/kg of cyanuric acid; and the MK100 group was given the basal diet supplemented with 100 mg/kg of melamine and 100 mg/kg of cyanuric acid. The melamine and cyanuric acid levels were analyzed in samples of feed by gas chromatography-tandem spectrometry (GC-MS/MS) in an accredited laboratory. The experimental period lasted for 40 days. Feed and water were supplied *ad libitum*.

At the end of the experiment, 8 birds per treatment group were randomly selected for a haematological and biochemical examination of blood. Blood was collected by puncturing the *vena basilica*.

Haematological indicators were determined in blood immediately after collection. The following haematological indicators were determined in whole blood: total red blood cell count (Er), haemoglobin concentration (Hb), haematocrit value (Ht), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH), mean cell volume (MCV), and total leukocyte count (Leu).

Biochemical indicators were determined in blood plasma, after centrifuging heparin-stabilized blood. Samples were analyzed using a DPC Konelab 20i analyzer® (Thermo Fisher Scientific Oy, Vantaa, Finland). The following biochemical indicators were determined: total protein (TP), glucose (Glu), cholesterol (Chol), uric acid (UA), aspartate aminotransferase (AST), calcium, phosphorus, magnesium, sodium, and potassium.

Statistical analysis was performed using STATISTICA CZ (Version 10, 2011) software. ANOVA was used to determine the differences in blood variables among the groups of broilers. When it showed significant differences between the groups, a Tukey's pair-wise comparison test was used. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The mean values of Er, Ht, Hb, MCHC, MCH, MCV, and Leu did not differ significantly ($P > 0.05$) among the respective groups (Table 2). However, in comparison to the control group, Er, Ht, Hb, and Leu values were lower in all experimental groups; the values determined in our study were similar to the results reported by Suchy et al. (2004).

Based on the results achieved in our study, it can be concluded that the decrease of red blood cell count is related to the administration of feed contaminated with melamine and cyanuric acid. Wang et al. (2010) state that in the case of the simultaneous contamination of diet with melamine and cyanuric acid, the insoluble complex of melamine-cyanurate is formed, which impairs the integrity of membrane of erythrocytes due to the decreased activity of Na^+/K^+-ATPase and due to the decreased membrane osmotic resistance against hypotonic pressure; it is possible to assume that their life span shortens. Furthermore, it can

Table 2. Haematological variables of broilers fed a particular diet

Item	Treatment[1]						
	C	M50	M100	K50	K100	MK50	MK100
Er (×10[6] cells/l)	2.77	2.66	2.21	2.31	2.76	2.59	2.420
	0.278	0.198	0.151	0.190	0.292	0.199	0.135
Haematocrit (%)	0.33	0.32	0.30	0.30	0.31	0.28	0.300
	0.030	0.021	0.010	0.013	0.014	0.011	0.014
Haemoglobin (g/l)	103.14	96.44	92.96	85.46	92.78	89.12	93.670
	6.537	4.054	4.336	2.732	3.210	4.365	4.609
MCHC (%)	31.67	31.02	30.69	28.65	29.8	31.88	31.120
	1.548	0.997	0.810	0.841	0.544	1.039	1.215
MCH (pg)	38.46	38.05	43.12	38.64	35.59	35.29	39.470
	2.418	4.155	2.859	3.074	2.805	2.010	2.684
MCV (fl)	122.67	124.22	140.62	135.4	119.09	112.22	126.990
	8.378	15.233	8.900	10.634	8.618	8.791	7.064
Leu (×10[9] cells/l)	22.00	17.63	17.88	18.13	17.38	20.19	16.940
	2.415	1.133	1.739	2.521	1.700	2.407	0.787

Groups: C = control; M50, M100 = basal diet + 50 (100) mg melamine/1 kg feed; K50, K100 = basal diet + 50 (100) mg cyanuric acid/1 kg feed; MK50, MK100 = basal diet + 50 (100) mg/kg melamine and 50 (100) mg/kg cyanuric acid/1 kg feed
Er = erythrocytes, MCHC = mean corpuscular haemoglobin concentration, MCH = mean corpuscular haemoglobin, MCV = mean cell volume, Leu = leukocytes
[1]mean ± SEM per animal (n = 8)

be assumed that the long-term administration of lower doses of melamine and cyanuric acid may have impact on the intrinsic haematopoiesis due to the lack of erythropoietin, as it is generally synthesized in kidneys. Impaired renal function in animals also results in azotemia and subsequent uremia. These conditions may subsequently result in erythrocytopenia (Doubek et al. 2010).

The values of Ht and Hb are influenced by the same factors as the red blood cell count, the lower values result in lower values of MCHC, MCH, and MCV. However, in our study, the MCV value moderately increased in the experimental groups M50, M100, K50, and MK100 and it can be concluded that not only the intrinsic red blood cell count was affected, but also the size of the cells (macrocytosis). Wang et al. (2010) reported that the incubation of erythrocytes with 400μM melamine–cyanurate complex resulted in deformation and aggregation of the cells.

Regarding the total leukocyte count, lower values were determined in all experimental groups as compared to the C group; it was markedly lower mainly in the MK100 group. It seems that the lower Leu count was caused again by the impairment of cell membrane integrity, whereas it could also be affected by the possible tissue destruction. Lu et al. (2012) found that especially kidney tissue is damaged, due to the formation of uroliths.

Lower concentrations of Hb, Ht, and Er count in the experimental groups in our study ($P > 0.05$) may also be related to the impairment of liver function due to insufficient synthesis of coagulation factors or lower protein synthesis. The lower Leu count in the experimental groups in our study ($P > 0.05$) can also be related to liver damage, due to negative impact of liver toxins on bone marrow (Doubek et al. 2010).

Table 3 shows mean values of biochemical blood indicators in all the evaluated groups in our study. The results show that there were highly significant ($P < 0.01$) differences in the content of TP, Glu, Ca, P, Na, and K, a significant difference ($P < 0.05$) was found in the content of Mg. Differences among Chol, UA, and AST values were not tested to be significant ($P > 0.05$).

The value of TP was lower in comparison to the C group in all the experimental groups in our study, which is in accordance with results reported by Brand et al. (2012); protein losses are often the sign of renal failure.

The value of Glu in our study was also lower in the experimental groups, the significant decrease

Table 3. Biochemical variables of broilers fed a particular diet

Item	Treatment[1]						
	C	M50	M100	K50	K100	MK50	MK100
TP (g/l)	$42.44^{B,b}$	$36.74^{A,B}$	$37.44^{A,B}$	$31.00^{A,B}$	$28.69^{A,B,a}$	27.65^{A}	$33.74^{A,B}$
	4.620	1.254	2.680	3.179	2.095	1.590	1.773
Glu (mmol/l)	$14.75^{B,b}$	$14.46^{B,b}$	$13.14^{A,B}$	$11.16^{A,B,a}$	9.95^{A}	9.99^{A}	$10.90^{A,B,a}$
	0.537	0.802	0.971	0.944	0.704	0.587	0.719
Chol (mmol/l)	2.91	3.50	3.85	3.20	2.68	2.79	3.36
	0.440	0.123	0.366	0.375	0.214	0.154	0.254
UA (μmol/l)	263.50	251.13	171.88	177.00	159.63	187.88	276.75
	74.086	54.376	25.989	44.615	37.107	41.681	40.230
AST (μkat/l)	6.12	7.71	8.89	5.95	7.61	5.58	7.12
	0.619	0.992	1.282	0.808	1.877	0.650	0.652
Ca (mmol/l)	3.09^{B}	3.05^{B}	$2.86^{A,B,b}$	$2.47^{A,B}$	2.17^{A}	$2.13^{A,a}$	$2.45^{A,B}$
	0.093	0.208	0.164	0.268	0.155	0.110	0.072
P (mmol/l)	$2.18^{A,B}$	$2.26^{A,B,b}$	2.52^{B}	$2.00^{A,B}$	1.81^{A}	$1.70^{A,a}$	1.77^{A}
	0.039	0.118	0.186	0.193	0.086	0.105	0.065
Mg (mmol/l)	1.22^{b}	$0.96^{a,b}$	$0.99^{a,b}$	$0.85^{a,b}$	0.75^{a}	0.73^{a}	$0.88^{a,b}$
	0.255	0.021	0.073	0.084	0.053	0.030	0.040
Na (mmol/l)	162.41^{B}	160.08^{B}	$154.71^{A,B,b}$	$133.95^{A,B}$	$121.04^{A,a}$	$120.20^{A,a}$	$136.85^{A,B}$
	1.779	4.550	9.630	11.460	7.513	4.958	4.541
K (mmol/l)	4.64^{B}	4.71^{B}	$4.13^{A,B}$	$3.81^{A,B}$	3.26^{A}	$3.68^{A,B}$	$4.14^{A,B}$
	0.204	0.240	0.295	0.375	0.222	0.151	0.164

Groups: C = control; M50, M100 = basal diet + 50 (100) mg melamine/1 kg feed; K50, K100 = basal diet + 50 (100) mg cyanuric acid/1 kg feed; MK50, MK100 = basal diet + 50 (100) mg/kg melamine and 50 (100) mg/kg cyanuric acid/1 kg feed
TP = total protein, Glu = glucose, Chol = cholesterol, UA = uric acid, AST = aspartate aminotransferase
^{A,B}means within a row with different superscripts differ ($P < 0.01$)
^{a,b}means within a row with different superscripts differ ($P < 0.05$)
[1]mean ± SEM per animal (n = 8)

($P < 0.01$; $P < 0.05$) was observed in the K50, K100, MK50, and MK100 groups as compared to the C group. Generally, the decrease of blood glucose level can be a sign of liver insufficiency. On the contrary, Brand et al. (2012) found the increase of blood glucose level when administering graded levels of melamine to broiler chickens. In our study, we also observed lower concentration of Ca in the experimental groups. Its highly significant ($P < 0.01$) decrease in comparison to the C group was confirmed in the K100 and MK50 groups. This is in agreement with the results reported by Suchy et al. (2014) who found lower plasma level of Ca in laying hens fed the melamine contaminated diet. Hypocalcemia often accompanies chronic renal failure, especially due to impaired vitamin D metabolism and increased losses of albumin in urine. In our case, it is necessary to consider the significantly lower values of TP in the K100 ($P < 0.05$) and MK50 ($P < 0.01$) groups, because hypoproteinaemia (or hypoalbuminaemia) results in lower value of TP due to the decreased binding capacity of Ca.

Regarding the concentration of P, we found its significant increase ($P < 0.01$; $P < 0.05$) in the groups of broilers fed diets contaminated with melamine only (M50 and M100 groups) as compared to the broilers fed diet contaminated with cyanuric acid alone (K100) or melamine and cyanuric acid in combination (MK50 and MK100).

Concerning the Na content, we observed a highly significant ($P < 0.01$) decrease in groups of broilers fed diet contaminated with cyanuric acid alone (K100) or diet contaminated with melamine and cyanuric acid in combination (MK50) as compared to the C group. Our results are in contrast with the results reported by Ding et al. (2012) who found the increasing blood concentration of Na with the increasing concentration of melamine and cyanuric acid in a diet. In renal failure, both, hypernatraemia or hyponatraemia may be present, whereas during acute renal failure, hyponatraemia is usually more frequent due to a polyuric phase.

A similar trend as in the case of the blood Na level was also observed in the Mg level; significantly lower ($P < 0.05$) values of Mg were found in the K100 and MK50 groups as compared to the C group.

As for the content of K, its lower value was found in broilers fed diet contaminated with cyanuric acid alone (K100) as compared to the C and M50 groups ($P < 0.01$); a non-significant ($P > 0.05$) decrease of its content was also found in the K50 and MK50 groups as compared to the C and M50 group ($P > 0.05$). Our findings are not in agreement with the results reported by Ding et al. (2012) who did not find any considerable changes in blood K level among particular groups of broilers in their experiment. During acute renal failure, there are often higher losses of K, which probably occurred also in our study in broilers from the K100, K50, and MK50 groups. However, in the final phase of chronic renal failure, the level of K is increased.

According to Puschner et al. (2007) and Dorne et al. (2013), melamine causes much higher nephrotoxicity in combination with cyanuric acid than if administered alone. Therefore it can be concluded that higher renal damage would cause considerable changes in biochemical blood profile, such as the decrease in the level of TP, Na, and/or Ca and K. Besides that, there can be changes in levels of haematological indicators, specifically the red blood cell count usually declines. This is in agreement with the results of our study; the most considerable changes in the above mentioned blood indicators were found in the groups of broilers fed the diet contaminated with the combination of melamine and cyanuric acid.

Hypoproteinaemia, hypoglycemia, hypokalemia, and hyponatraemia observed in broilers fed diets with the higher level of melamine and cyanuric acid (alone or in combination) in our study can be a sign of gradual liver failure. Gao et al. (2010) found in ducks that administration of higher doses of melamine in feed caused also histological lesions in the liver.

As compared to the C group, the activity of AST enzyme in our study was higher in the experimental groups ($P > 0.05$), with the exception of the K50 and MK50 groups. However, the AST level is a nonspecific indicator of liver failure because its higher level is detected also when myocardium or skeletal muscles are damaged. Furthermore, the activity of AST also increases when erythrocytes are damaged (Doubek et al. 2010). Non-significant ($P > 0.05$) changes in the level of Chol were found between the experimental groups and C group in our study; similar values for Chol in broiler chickens were reported by Rouckova et al. (2004) and Herzig et al. (2009).

CONCLUSION

The concentrations of melamine and cyanuric acid used in the contaminated diets for broiler chickens resulted in significant ($P < 0.01$; $P < 0.05$) changes in the content of TP, Glu, Ca, P, Mg, Na, and K in their blood plasma. In comparison to the control group, these changes were most noticeable in broilers fed diets contaminated with cyanuric acid only (K50 and K100), or diets contaminated with melamine and cyanuric acid in combination (MK50 and MK100).

Based on the results of our study, it can be concluded that not only renal function was impaired when diet was contaminated with melamine and cyanuric acid alone or in combination, but also the liver, and/or erythrocytes and leukocytes, could be partially damaged.

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