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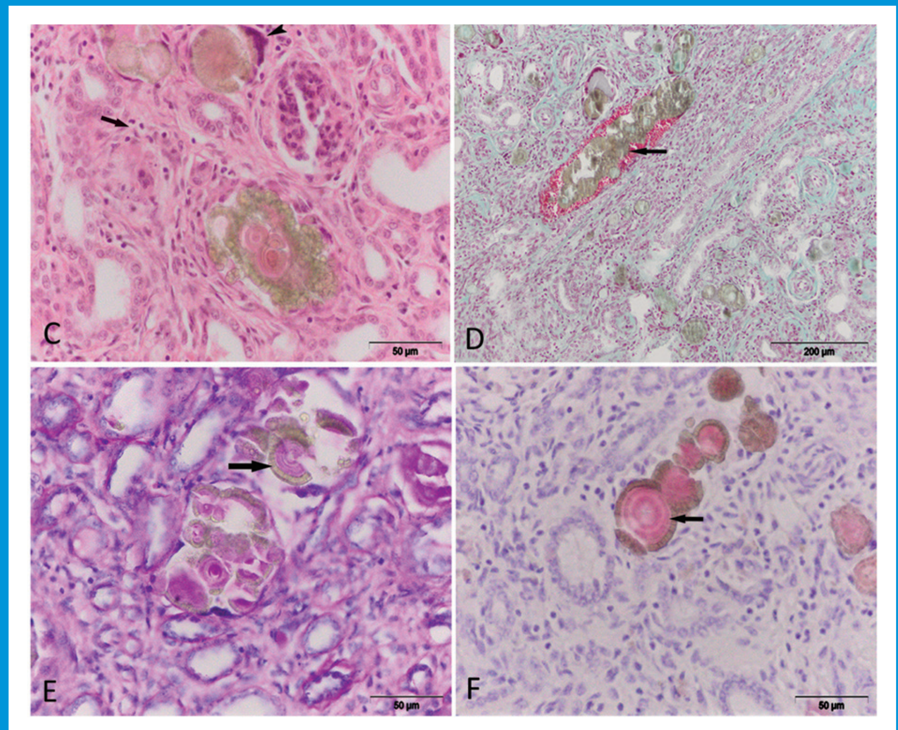

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Original Scientific Article

OXIDATIVE STRESS RESPONSE IN LIVER OF BROILER CHICKENS SUPPLEMENTED WITH N-3 PUFA-RICH LINSEED OIL

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Summary: The aim of this study was to investigate the oxidative stress response, at the transcriptional level, in chickens supplemented with n-3 polyunsaturated fatty acids (PUFAs). Twenty chickens were divided into two groups: the PALM group (N=10) received 5% palm oil in feed as a source of saturated fatty acids (SFA), while the LIN group (N=10) received 5% polyunsaturated linseed oil. We determined plasma and liver malondialdehyde concentrations that served as a marker of lipid oxidation and vitamin E, as a natural antioxidant. Additionally, plasma triglyceride and cholesterol concentrations and liver fatty acid (FA) composition were determined. The RNA was isolated from the liver, and a whole-chicken genome microarray analysis (Affymetrix) was performed to examine the expression of genes. Differential expression of selected candidate genes was confirmed using quantitative real-time polymerase chain reaction (qRT-PCR). Malondialdehyde concentration was higher and vitamin E concentration was lower in the LIN group. No differences in plasma triglyceride and cholesterol concentrations were observed. Liver FA composition reflected the FA composition of the diets. Clearly present prooxidative conditions due to the consumption of an n-3 PUFA-rich diet triggered an oxidative stress response through the up-regulation of *NFE2L2* and *PIK3R1* genes. Changes in liver transcriptome also suggest that PUFAs lower mitochondrial lipid oxidation and increase the degree of lipogenesis in chickens' livers.

Key words: chicken; linseed oil; palm oil; lipid metabolism; gene expression

Introduction

In the poultry industry, various fat sources are used to meet the high-energy requirements of broiler chickens. Such sources used can be mixtures or sole sources of saturated (animal fat, palm oil, palm kernel oil) or unsaturated (oils of plant origin) fatty acids (FAs), depending on the availability and price of the fat source in different parts of the world. The saturation or unsaturation of fat used in chicken diets affects the FA composition of chicken tissues and thus has an impact on the nutritional value of such products for human nutrition. This fact has led to the deliberate manipulation of chicken diets to obtain products of higher nutritional value: for example, meat products rich in n-3 polyunsaturated fatty acids (PUFAs), which have been associated with several health benefits for humans (1). However, it is often ignored that the inclusion of fat sources rich in unsaturated FAs increases the peroxidizability of PUFA-enriched animal products (2). The exposure of such FAs to increased levels of oxidants in the organism also

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generates lipid peroxides that play an important factor in shortening the shelf life of meat as well as affecting organoleptic properties, such as rancidity and meat discoloration (3).

Fatty acids, FA-Coenzyme As or FA metabolites can induce a cascade of events leading to a covalent modification of transcription factors (4). The mechanisms whereby n-3 FAs affect gene expression are complex and involve multiple processes, including transcription factors, such as sterol regulatory-element binding proteins (SREBPs) and peroxisome proliferator activated receptors (PPARs), which are critical for modulating the expression of genes controlling both systemic and tissue-specific lipid homeostasis (5). In addition to FAs, reactive oxygen species (ROSs), which cause damage to biomolecules such as DNA, proteins, and lipids, can also regulate the expression of redox-sensitive genes (6).

Although we and other authors have clearly demonstrated that n-3 PUFA-rich diets increase oxidative susceptibility in chicken tissues and affect the shelf life of meat products (7), no study has yet investigated the response to oxidative stress at the transcriptional level in chickens fed an n-3 PUFA-rich diet. Furthermore, differences in the expression of genes involved in lipid and cholesterol metabolism were also of interest in the study. This knowledge is essential in order to understand the mechanisms that underlie the biological effects of PUFAs in chickens from the perspectives of animal welfare and using such products for human consumption.

Material and methods

Twenty one-day-old male broiler chickens of type Ross 308 were housed in two groups in floor pens at a temperature of 30 °C (gradually decreasing as the animals grew) in conditions consisting of 16 h of light and 8 h of dark for 30 days. Diets based on wheat and soybean meal were formulated according to broiler nutrition specifications for the Ross 308 (Aviagen, 2007) and differed only in the source of added fat, which was either palm oil in the PALM group (N=10) or linseed oil in the LIN group (N=10). Linseed oil and palm oil were used to obtain the most evident distinction in oxidative stress susceptibility between the treatment groups. The amount of added fat was 50 g/kg of feed mixture. Groups were supplemented with 10 mg/kg of α-tocopheryl acetate to meet NRC (1994) requirements for chickens. This amount was lower than in Ross 308 nutrition specifications in order to induce oxidative stress in the LIN group. Linseed oil was purified of vitamin E by using the deodoration process to reduce the effect of vitamin E present in the oil. Feed mixtures were prepared fresh every 10 days at the departmental feed mill, stored at -20 °C and thawed on the day of feeding. After 30 days, the animals were sacrificed by cervical dislocation followed by exsanguination and all the blood and liver were collected for analyses. All the animal experiments were performed in the experimental facilities of the Animal Science Department of the Biotechnical Faculty, University of Ljubljana, in accordance with institutional guidelines, and were approved by the Animal Ethics Committee of the Ministry for Agriculture, Forestry and Food and by the Veterinary Administration of the Republic of Slovenia.

Plasma was obtained by centrifugation (1000 × g, 15 min at 4 °C), transferred to Eppendorf tubes and stored at -70 °C. Thin tissue slices of liver intended for gene expression analyses were snap-frozen in liquid nitrogen and kept at -70 °C. Plasma samples were analysed for the determination of malondialdehyde (MDA) and vitamin E using HPLC (Waters, Milford, USA) equipped with a Waters 474 scanning fluorescence detector. The fatty acid composition of samples was analyzed using a gas chromatographic method following the transesterification of lipids, as previously described by Fidler et al. (8). Total cholesterol and triglyceride concentrations were determined with an RX Daytona automated biochemical analyser, using cholesterol CH 3810 and triglyceride TR 3823 kits (Randox, Crumlin, UK).

The pulverized liver tissue was homogenized in TRI-reagent (Sigma, Germany) with Ultra turrax (IKA, Labortechnik, Germany), and RNA was extracted according to the manufacturer's instructions. The RNA samples of four birds per treatment were selected for gene expression analysis on an Affymetrix GeneChip® Chicken Genome Array (900592, Affymetrix, Santa Clara, USA). The RNA preparation, hybridisation and scanning were performed at ARK Genomics, the Roslin Institute, University of Edinburgh, following the standard protocols (Affymetrix GeneChip® Expression Analysis Technical Manual). Differentially expressed genes from the important

pathways were selected for validation by the qRT-PCR. A sample (1 μg) of total RNA was treated with amplification grade DNase I, and reverse transcribed with a SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, California, USA). Real-time quantitative PCR was performed on the LightCycler 480 detection system using LightCycler® 480 SYBR Green I Master according to the manufacturer's instructions (Roche, Mannheim, Germany). Primers were designed to span introns or bridge an exon-exon junction, using Primer3 software. Melt curve and standard curve analyses were performed to determine the specificity and efficiency of primer pairs. The PCR was performed with the following PCR parameters: 95 °C for 10 min, then 95 °C for 10 s, 60 °C for 30 s, 72 °C for 5 s for 38 cycles plus a dissociation step (60–95 °C). The relative expression ratios were calculated as described (9), using stably expressed genes *Rpl4, Rplp0, Ppib and Eif2a* as internal references.

Statistical analysis was performed using the group as the main effect (SAS/STAT module SAS 8e, 2000; SAS Inc., Cary, NC, USA). Differences were determined based on the Tukey-Kramer multiple comparison test and were considered to be significant at $P<0.05$. Results in the tables are presented as least square means (LS-means) ± SEM with P-values. The data normalization and statistical analysis of gene expression (ANOVA) was carried out with Partek Genomics Suite software (Partek Inc., St. Louis, Missouri, USA). The genes with $P<0.05$ and a simultaneously intensity of a fold change higher than 1.2, were considered to be differentially expressed. For further bioinformatics analysis, the gene lists were processed using Ingenuity Pathways Analysis Software (Ingenuity Systems, Redwood City, Ca, USA) to identify the relationships, mechanisms, functions, and pathways of relevance of differentially expressed genes.

Results

The chickens remained healthy during the experiment; there were no differences in body weight, but the chickens from the PALM group had a higher feed consumption ($P<0.05$, data not shown). Liver fatty acid composition differed between groups as demonstrated by higher proportions of PUFAs and monounsaturated FAs and lower proportions of SFAs in the LIN group (Table 1). The proportions of n-3 PUFAs (C18:3 n-3, C20:5 n-3 and C22:6 n-3) were higher in the LIN group and the ratio between n-6 and n-3 PUFAs in this group was in favour of n-3 PUFAs.

Table 1: The liver fatty acid composition of chicken supplemented with 5% palm or linseed oil (wt% of total fatty acids)

	PALM	LIN	SEM	P-value
C18:1 n-9	24.5	14.6	1.2	<0.01
C18:2 n-6	16.4	16.9	0.6	0.53
C18:3 n-3	0.376	7.80	0.559	<0.01
C20:4 n-6	8.96	3.79	0.52	<0.01
C20:5 n-3	0.851	8.05	0.120	<0.01
C22:6 n-3	1.78	4.93	0.26	<0.01
SFA	39.4	37.0	0.5	<0.01
MUFA	28.7	16.6	1.4	<0.01
PUFA	31.9	46.4	1.3	<0.01
n-3 PUFA	3.65	24	0.50	<0.01
n-6 PUFA	28.2	22.3	1.01	<0.01
n-6/n-3	8.08	0.930	0.367	<0.01

[1]Values are the means of 10 animals per group; PALM = 5 % palm oil, LIN = 5 % linseed oil

[2]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 2: Plasma and liver malondialdehyde and vitamin E concentrations, and plasma cholesterol and triglyceride levels

	PALM	LIN	SEM	*P*-value
Plasma MDA (nmol/ml)	0.281	0.734	0.041	<0.01
Liver MDA (nmol/g)	0.877	1.62	0.083	<0.01
Plasma α-tocopherol (μg/ml)	8.19	5.79	0.52	<0.01
Plasma γ-tocopherol (μg/ml)	0.501	0.290	0.021	<0.01
Liver α-tocopherol (μg/g)	11.71	6.92	0.71	<0.01
Liver γ-tocopherol (μg/g)	0.829	0.475	0.057	<0.01
Plasma cholesterol (μmol/ml)	3.23	3.45	0.12	0.203
Plasma triglycerides (μmol/ml)	0.231	0.224	0.019	0.796

[1]Values are the means of 10 animals per group; PALM = 5 % palm oil, LIN = 5 % linseed oil

Table 3: Differentially expressed genes involved in oxidative stress response (LIN vs PALM)

Gene Symbol	Gene Title	Microarray fold change1	RT-qPCR fold change2	RT-qPCR P-value2
OSGIN1	Oxidative stress induced growth inhibitor 1	4.32**	3.76	0.01
HNF4A	Hepatocyte nuclear factor 4, alpha	3.15**	2.02	0.26
COQ10B	Coenzyme Q10 homolog B (S, cerevisiae)	-3.02**	-2.91	0.03
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1	1.86**	1.72	0.06
NFE2L2	Nuclear factor (erythroid-derived 2)-like 2	1.73**		
ATF4	Activating transcription factor 4	1.70*		

1 **P-value<0.01, *P-value<0.05; 2Only for genes validated by the qRT-PCR; 3Values are the means of 4 animals per group. LIN = 5 % linseed oil (source of PUFA); PALM = 5 % palm oil (source of SFA).

Table 4: Differentially expressed genes involved in lipid and cholesterol metabolism (LIN vs PALM)

Gene Symbol	Gene Title	Microarray fold change1	RT-qPCR fold change2	RT-qPCR P-value2
DIO3	Deiodinase, iodothyronine, type III	-20.03**	-16.88	0.09
CYP2C45	Cytochrome P-450 2C45	10.01**	18.03	0.11
DIO2	Deiodinase, iodothyronine, type II	8.80**		
SIK1	Salt-inducible kinase 1	-4.82**	-4.64	0.05
FDFT1	Farnesyl-diphosphate farnesyltransferase 1	4.43**	5.92	0.17
LPIN1	Lipin 1	-4.30**		
PDK4	Pyruvate dehydrogenase kinase	-4.28**	-4.15	0.03
MID1IP1	MID1 interacting protein 1	2.64**		
HMGCL	3-hydroxymethyl-3-methylglutaryl-CoA lyase	-2.50**	-2.15	0.19
INSIG	Insulin induced gene 1	-2.49*		
HMGCR	3-hydroxymethyl-3-methylglutaryl-CoA reductase	1.82*		
SIRT1	Sirtuin	-1.74**		
PPARA	Peroxisome-proliferator-activated receptor α	-1.57**	-1.37	0.20

1 **P-value<0.01, *P-value<0.05; [2]Only for genes validated by the qRT-PCR; [3]Values are the means of 4 animals per group. LIN = 5 % of linseed oil (source of PUFA); PALM = 5 % of palm oil (source of SFA)

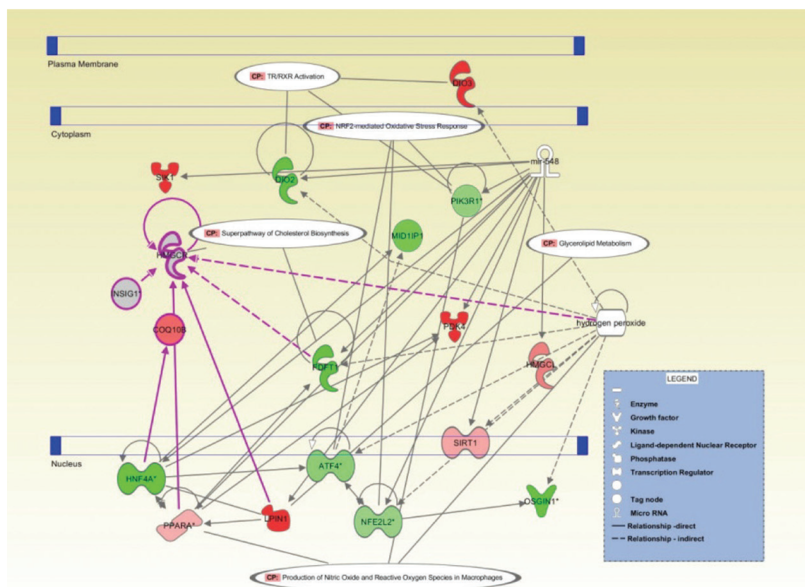

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Figure 1: Relationships of differentially expressed genes involved in oxidative stress response and lipid metabolism genes along with overlaid functional information

Pathway analysis of differentially expressed genes involved in oxidative stress response and lipid / cholesterol metabolism between the LIN and PALM groups. Nodes represent genes, solid lines represent characterized direct gene relationships, and broken lines indirect gene relationships based on the literature and Ingenuity knowledge database. The node shapes denote the activity or molecular function of a particular gene (see legend). The intensity of the node colour indicates the degree of differential expression: red and green represents decreased and increased expression in LIN versus PALM, respectively. For fold changes, please refer to Tables 3 and 4.

Oxidative stress in the body was evaluated by means of plasma and liver MDA. Their concentrations were higher in the LIN group (Table 2). Regarding vitamin E, only the content of α- and γ-tocopherol were considered in the analysis and interpretation, as the concentrations of β- and δ-tocopherol were at the lower detectability limit for the method used. The content of measured tocopherols was higher in PALM group, there were no differences in plasma cholesterol and triglyceride levels (Table 2).

In the microarray experiment, a comparison of the groups showed 1157 differentially expressed genes ($P<0.05$ and at least 1.2 fold change). The genes that were differentially expressed were involved in lipid and carbohydrate metabolism, cell signalling, gene expression and oxidation-reduction processes (Ingenuity Pathways Analysis). The results of the pathway analysis for differentially expressed genes involved in oxidative stress response and lipid metabolism are shown in Tables 3 and 4. The direct and indirect relationships of these genes along with overlaid functional information are displayed schematically in Figure 1. The key genes from these pathways were chosen for qRT-PCR validation. Due to the inter-individual variability and small sample size, the expression of some genes did not meet statistical significance in the qRT PCR experiment. However, all of the selected genes (Tables 3 and 4) showed the same direction of change in the expression (up- or down-regulation). Additionally, similar fold change differences in the qRT-PCR in comparison to the microarray results support its validity.

Discussion

A high PUFA diet increases the risk of oxidative stress (10). Fébel et al. (11) evaluated the effect of dietary PUFAs in broilers and found a significant increase in erythrocyte and liver MDA level. Our results support these findings, as plasma and liver MDA were both elevated in the group receiving linseed oil. Dietary linseed oil induced the expression of *PIK3R1* (phosphoinositide-3-kinase) and *NFE2L2* (nuclear factor (erythroid-derived 2 -like 2) (Table 3). *NFE2L2* is of particular functional importance for the present study as this gene has been shown to be a main transcription factor regulating the expression of genes involved in detoxification and elimination of ROS. In mice, a knockout of *NFE2L2* leads to increased sensitivity to oxidative stress in a variety of organs and cells (12). Its dimerization partner, activating transcription factor 4 (*ATF4*), was also up-regulated in the LIN group (Table 3). Nuclear transition of NRF2 and its binding to antioxidant response element is regulated by PIK3R1 (13). The increased expression of oxidative stress-induced growth inhibitor 1 (*OSGIN1*) in LIN should also be induced through the activation of NRF2, as it is

known that oxidized phospholipids stimulate its expression (14). Dietary linseed oil also increased the expression of *HNF4A*, which has been shown to stimulate lipid peroxidation and reduce antioxidant defence in Caco-2 cells (15). Due to the lack of functional studies on differentially expressed genes in chickens, a pathway analysis (Figure 1) of *NFE2L2* in humans was performed, using Ingenuity software. As shown in Figure 1, many proven functional links exist between *NFE2L2* and its function in oxidative stress and inflammatory response in the liver. Therefore, the present study's results of increased expression of *NFE2L2* in the LIN group suggest that it plays an important role in these biological processes in the chicken as well.

The hepatic lipid metabolism is a highly co-ordinated process, in which many processes are regulated by nuclear receptors and transcription factors. In birds, liver is also the main site of *de novo* lipogenesis (16). Regulation of gene transcription by FA is due to changes in the activity of at least four transcription factors families: PPAR, LXR (liver X receptor), HNF-4α (hepatic nuclear factor 4, alpha) and SREBP (17). Fatty acids themselves are ligands for PPARα and, as such, activate its transcriptional activity (18). PPARα regulates a set of enzymes that are crucial for FA oxidation, and increases the transcription and expression of proteins and enzymes necessary to transport and catabolise FA (17). Its transcription is influenced by lipin 1 (LPIN1) (19). The expression of *LPIN1* and *PPARA* was lower in the group receiving linseed oil, which implies a lower degree of mitochondrial lipid oxidation. PPARα activation was also shown to lower the cholesterol concentration by reducing the abundance of SREBF-2 (20). However, this study was performed in rats and in the rat hepatoma cell line after stimulation with PPARA agonists, and hence may not be directly comparable to the present in vivo study in the chickens.

In the present study, dietary linseed oil up-regulated *SREBF2* 1.40-fold (Table 4). Among the genes regulating cholesterol biosynthesis, *FDFT1* (farnesyl-diphosphate farnesyltransferase) and *HMGCR* (3-hydroxymethyl-3-methylglutaryl-CoA reductase) were also up-regulated in LIN. Simultaneously, *HMGCL* (3-hydroxymethyl-3-methylglutaryl-CoA lyase), the gene encoding an enzyme involved in ketone bodies production was down-regulated in LIN (Table 4). Although the effect of linseed oil on expression of genes involved in liver cholesterol synthesis was clearly detected, these changes did not modify its plasma levels. It is possible that a 30-day treatment was not long enough or some other compensatory mechanisms took place, such as changes in reverse cholesterol transport or excretion rate of cholesterol via bile.

The degree of lipogenesis in the liver is also influenced by PUFAs. Cytochrome P-450 2C45 gene (*CYP2C45*), which plays an important role in lipid metabolism by regulating the availability of PUFAs and their metabolites (21), was up-regulated in LIN. Salt-inducible kinase gene (*SIK1*) was down-regulated by the addition of linseed oil, which is in accordance with previous findings (22). The Mid1 interacting protein 1 gene (*MID1IP1*), involved in the stimulation of hepatic lipogenesis by LXR ligand treatment (23), was also up-regulated in the LIN group. As regards the expression of genes involved in the activation (deiodinase type II – *DIO2*) or deactivation (deiodinase type III – *DIO3*) of thyroid hormones, *DIO2* was up-regulated in LIN and *DIO3* was down-regulated. This is in agreement with the study from Ferrini et al. (24) who observed higher T3 concentrations in chickens fed a 5% linseed oil diet than in those fed beef tallow. Thyroid hormones may thus be affected by dietary fat composition and play a role in lipid metabolism in poultry.

Based on our nutritional challenge of broiler chickens with linseed (PUFA-enriched) oil and evaluation of liver transcriptome, we conclude that significant changes occurred in the expression of the oxidative stress response and lipid and cholesterol metabolism genes. We demonstrate for the first time, that, similar to mammals, the oxidative stress response in the chickens is also mediated by up-regulation in expression of *PIK3R1* and *NFE2L2* genes. Other changes in liver transcriptome also suggest that PUFAs lower mitochondrial lipid oxidation, which may impair metabolic flexibility in response to lipid exposure as shown for obese humans (25). Furthermore, gene expression in the liver suggested increased lipogenesis in the chicken liver in the LIN group and hence increased susceptibility to hepatic steatosis as shown in other poultry breeds (26). Our study, therefore, emphasises the need for more targeted nutritional research into poultry systems that aim at genetic or nutritional manipulation of body composition to better define nutritional specification (e.g., oxidative protection) in the diets.

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ODZIV NA STANJE OKSIDACIJSKEGA STRESA PRI PITOVNIH PIŠČANCIH, KRMLJENIH Z LANENIM OLJEM

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Povzetek: V raziskavi smo preučevali kakšen je odgovor na oksidacijski stres na ravni traskriptoma pri piščancih, krmljenih z večkrat nenasičenimi maščobnimi kislinami (VNMK). Dvajset piščancev smo razdelili v dve skupini. Skupina PALM (N=10) je prejemala 5 % palmove maščobe, ki je dober vir nasičenih maščobnih kislin (MK), skupina LIN (N=10) pa laneno olje, ki vsebuje velik delež večkrat nenasičenih MK. V plazmi in jetrih smo določili koncentracijo malondialdehida (MDA), ki je pokazatelj lipidne peroksidacije v organizmu, in vitamina E, ki je naravni antioksidant. V plazmi smo izmerili še koncentracijo trigliceridov in holesterola, v jetrih pa ugotovili vrste MK. Iz jeter smo izolirali RNK in z analizo mikromrež (Chicken Genome Array Affymetrix) določili izražanje genov celotnega genoma. Rezultate izbranih diferencialno izraženih genov smo potrdili s PCR v realnem času. Lipidna peroksidacija je bila večja v skupini LIN, kjer smo namerili tudi manj vitamina E kot v skupini PALM. Razlik v koncentraciji trigliceridov in holesterola ni bilo. Maščobno-kislinska sestava v jetrih je bila v skladu z MK v krmi, ki so jo piščanci prejeli. Prooksidativno stanje, povzročeno z zauživanjem n-3 VNMK, je izzvalo odgovor na oksidacijski stres preko povečane izraženosti genov *NFE2L2* in *PIK3R1*. Različno izraženi jetrni geni nakazujejo vpliv VNMK na manjšo oksidacijo MK in večjo lipogenezo v jetrih.

Ključne besede: piščanci; laneno olje; palmova maščoba; presnova maščob; izraženost genov

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Original Scientific Article

MALASSEZIA, MITES AND BACTERIA IN THE EXTERNAL EAR CANAL OF DOGS AND CATS WITH OTITIS EXTERNA

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Summary: Occurrence of *Malassezia*, mites and bacteria, was evaluated through cytology, culture and microscopical analysis of auricular cerumen collected from 115 cats and 203 dogs with otitis externa. For the identification of *Malassezia* species, a PCR-based technique was also used. All the patients enrolled in the study were examined for *Malassezia* and all cats and 101 dogs were also investigated for mites. Bacteriological examination was performed on 16 cats and 60 dogs. The associations between *Malassezia* and the other pathogens and the correlations between *Malassezia* and season, gender and ear conformation, were evaluated. *Malassezia* pachydermatis was isolated from 58.2% cats and 52.7% dogs, while *Otodectes cynotis* was identified in 66.1% cats and in 5.9% dogs. Bacteria were detected in 18.7% cats and 36.7% dogs and *Staphylococcus pseudointermedius*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Streptococcus canis*, *Escherichia coli* and *Bacillus* sp. were identified. *M. pachydermatis* was isolated in pure culture from 28.3% cats and from 87.1% dogs, while it was associated with *O. cynotis* in 70.1% of cats and in 5% of dogs, and with bacteria in 1.5% of cats and 23.3% of dogs. Mixed infections caused by *M. pachydermatis*, bacteria (*S. pseudointermedius*) and mites occurred in 1 cat and 1 dog. Our results suggest that ear conformation is an important individual predisposing factor for *Malassezia* otitis in dogs and indicated the influence of the season on onset of *Malassezia* infections in cats.

Key words: *Malassezia pachydermatis*; *Otodectes cynotis*; bacteria; otitis externa; dog; cat

Introduction

Otitis externa is a common presenting complaint in veterinary medicine, both in general and referral practices. It is also one of the more frustrating entities practitioners are called on to deal with (1). Yeasts belonging to *Malassezia* genus, mites such as *Otodectes cynotis* and bacteria, mainly *Staphylococcus* spp., *Streptococcus* spp., *Proteus* spp., *Pseudomonas* spp., *Escherichia coli*, are frequently involved in the occurrence of otitis externa (2, 3, 4). Mites are regarded as primary causes and are reported to occur in up to 50% of cats and 5-10% of dogs (2), while yeasts and bacteria are not considered primary pathogens, mainly acting as perpetuating or predisposing factors (2, 3, 5, 6, 7). However, in the literature data about a possible association among these pathogens are scarce.

Successful management of otitis externa mainly depends on the understanding of the primary, predisposing, and perpetuating factors involved in its pathogenesis (2, 5). These factors cannot be grossly distinguished from each other and are frequently involved in mixed infections

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(2, 5, 7). Individual predisposing factors could also play an important role, working in concert with the primary or perpetuating causes of otitis externa in causing clinical disease (2).

The aim of this retrospective study was to assess the occurrence of yeasts, mites and bacteria in cats and dogs affected by otitis externa, evaluating associations among the occurrence of *Malassezia* and other pathogens, and possible correlations of *Malassezia* infections with season and individual factors, such as gender and ear conformation.

Material and methods

Adult feline and canine patients (more than 1 year old) referred for otitis externa to private veterinary practitioners were enrolled in the study. Patients that received specific otologic drugs 2 months prior to the referral were excluded. Diagnosis had to be accomplished on the basis of clinical signs such as head shaking, ear scratching, auricular discharge, malodour, erythema, ear swelling and pain (20). Breed, gender and season in which otitis presented had to be available for data analysis.

Table 1: Number and breed of dogs with otitis externa examined for *Malassezia*, mites and bacteria

Dog breed	N. animals
Alan	1
Basset Hound	1
Border Collie	1
Boxer	7
Cocker Spaniel	6
Course	1
Dalmatian	1
English Setter	7
Espagneul Breton	1
German Sheperd	5
Golden Retriever	1
Italian Pointer	1
Italian Bloodhound	1
Labrador Retriever	32
Maremma Shepherd	1
Newfoundland	1
Pekingese	2
Pug	3
Puli	1
Shar-pei	1
Springer Spaniel	1
St. Bernard	1
West Highland White Terrier	1
Cross-breed	125
Total	**203**

All cats were European shorthair, while cross-bred dogs represented the majority (125/203) as reported in Table 1. Ear conformation was recorded for 178 dogs (29 straight and 149 pendulous). Gender was recorded in 49 (24 females, 25 males) cats and in 198 (97 females, 101 males) dogs. The season of onset of clinical signs was reported in 76 cats and 203 dogs.

All the animals were examined for *Malassezia*, all cats and 101 dogs were also investigated for *O. cynotis*. Bacteriological examinations were performed on 16 cats and 60 dogs, respectively. From both ears of all examined animals, samples of ear wax and secretions were collected by means of sterile ear swabs. All samples were examined for *Malassezia* by cytology. Otologic smears were stained by Diff Quick® (Diff-Quik, Medion Diagnostics AG, Düdingen, Switzerland) and examined at 400X and 1000X. Diagnosis of *Malassezia* otitis was achieved when more than 10 blastospores/microscopic field (mean number of 10 fields) at 400X were observed (8). On positive samples, culture onto m-Dixon medium was performed as previously described (9, 10). Briefly, after collection, the specimens were promptly seeded onto Sabouraud dextrose agar added with 0.5% of chloramphenicol and cycloheximide (Sabouraud Agar + Actidione®, Liofilchem, Italy) and mDixon Agar (3.6% malt extract, 0.6% peptone, 2% desiccated ox-bile, 1% Tween 40, 0.2% glycerol, 0.2% oleic acid, 1.2% agar, 0.5% chloramphenicol and 0.5% cycloheximide). All the plates were incubated at 30 °C for about 7 days, and daily inspected for *Malassezia* growth from day 4 post-inoculation.

Preliminary identification of yeasts was based both on macroscopic appearance of colonies and microscopic cell morphology. Bond and Anthony (11) demonstrated the possible lipid dependence of some isolates of *M. pachydermatis*, so strains referable to this species apparently lipid-dependent were identified by serial transfers on a lipid-free culture medium. The Tween assimilation test as described by Guillot et al. (12) and catalase activity were performed as additional tests to both to confirm the identification, and to exclude the presence

of other *Malassezia* species. Morphological and biochemical identification was confirmed by means of a PCR-based technique using restriction enzyme digestion, specific for the discrimination of 11 *Malassezia* species, as described by Mirhendi et al. (13). In order to achieve pure cultures, five colonies of each positive sample were subcultured onto mDixon Agar and stored at −20 °C until analysis. The cell walls were mechanically disrupted by freeze–thawing and genomic DNA was extracted and purified according to the DNeasy™ protocol for animal tissue (QIAGEN Inc., Valencia, CA, USA). The primers selected for this protocol amplify the target part of 26S rDNA, providing a single PCR product of an expected size of 580 bp. The PCR products were subjected to REA using CfoI and BstF51, separately, according to the manufacturer's instructions (Fermentas International Inc., Burlington, Ontario, Canada). Digested fragments were analyzed by electrophoresis in 2% agarose gel.

Otologic smears from 16 cats and 60 dogs were examined also for bacteria, both by cytology and culture. At cytology, bacterial population (rods and cocci) was considered overrepresented when more than 6 organisms at 1000X (mean number of 10 fields) were detected in Diff-Quik stained otologic smears (3). Samples were cultured on Blood Agar and incubated aerobically at 37°C for 24 hours. If bacterial growth occurred, the different colonies were submitted to Gram staining, then cultured on plates containing the following selective media: Tryptone Bile X-glucoronide Medium (Oxoid LTD, Basingstoke, Hampshire, England), *Pseudomonas* CFC Selective medium (Oxoid), Baird-Parker Medium + Egg Yolk Tellurite Emulsion (Oxoid), Violet Red Bile Agar (Oxoid). Plates were incubated aerobically at 37°C for 24 hours. The isolates obtained were typed by API System 20E, API System 20NE, API System Staph, API System 20 Strep (BioMerieux, Marcy-l'Etoile, France). For the search of mites, ear wax from all cats and 101 dogs was microscopically examined at 100X and 400X.

Data were statistically elaborated with χ^2 and ANOVA tests (14) in order to evaluate correlations of *Malassezia* occurrence with season, gender, ear conformation and the possible association among the different pathogens examined (significativity $P<0.05$).

Results

Cytological examinations allowed to detect more than 10 yeast cells/field from 67/115 (58.2%) cats and 107/203 (52.7%) dogs. On the basis of culture results, *Malassezia pachydermatis* was identified in pure culture in all positive specimens. *O. cynotis* was identified in 76/115 (66.1%) cats and in 6/101 (5.9%) dogs. Bacteria were detected in 3/16 (18.7%) cats and from 22/60 (36.7%) dogs and more than 6 organisms at 1000X were found in all 25 positive samples. More detailed results are presented in Table 2. Cultures allowed the identification of *Staphylococcus pseudointermedius* (1 cat and 10 dogs), *Staphylococcus epidermidis* (6 dogs), *Pseudomonas aeruginosa* (3 dogs), *Streptococcus canis* (1 cat and 1 dog), *E. coli* (1 cat and 1 dog) and *Bacillus sp.* (1 dog) (Table 3).

Table 2: Prevalence of *Malassezia pachydermatis*, *Otodectes cynotis* and bacteria in 203 dogs and 115 cats with otitis externa

	Malassezia pachydermatis		
	Examined	Positive	Prevalence
Dogs	203	107	52.7%
Cats	115	67	58.2%
	Otodectes cynotis		
	Examined	Positive	Prevalence
Dogs	101	6	5.9%
Cats	115	76	66.1%
	Bacteria		
	Examined	Positive	Prevalence
Dogs	60	22	36.7%
Cats	16	3	18.7%

Table 3: Prevalence of bacteria species isolated from 60 dogs and 16 cats with otitis externa

	Prevalence (positive/examined) in dogs	Prevalence (positive/examined) in cats
Staphylococcus pseudointermedius	16.7% (10/60)	6.3% (1/16)
Staphylococcus epidermidis	10% (6/60)	0/16
Pseudomonas aeruginosa	5% (3/60)	0/16
Streptococcus canis	1.7% (1/60)	6.3% (1/16)
Escherichia coli	1.7% (1/60)	6.3% (1/16)
Bacillus sp.	1.7% (1/60)	0/16

M. pachydermatis was isolated as unique agent from 19/67 (28.3%) cats and from 88/101 (87.1%) dogs, while it was associated with *O. cynotis* in 47/67 (70.1%) cats and in 5/101 dogs (5%). Yeasts and bacteria were associated in 1/16 (6.2%) cats and in 14/60 (23.3%) dogs. *O. cynotis* was isolated as unique agent in 29/76 (38.2%) cats, while mites alone were never recovered from dogs. Bacteria as sole causative agents were observed in 7/22 (31.8%) dogs and 0/3 cats. Mixed infections caused by yeasts, bacteria (*S. pseudointermedius*) and mites occurred in 1 cat (6.2%) and 1 dog (1.7%), respectively.

Significant statistical values were found when comparing dog ear conformation and occurrence of *Malassezia* (χ^2=4.69, $P < 0.05$), with significantly frequent infections observed in dogs with pendulous ears. A seasonal dependence ($P < 0.05$) was also observed in the onset of *Malassezia* infection in cats that was mostly recorded in summer and winter (evaluated by ANOVA test).

Discussion

One of the aims of the present study was to investigate the occurrence as well as correlation between *Malassezia* infection and the presence of other agents of otitis externa in cats and dogs, such as bacteria and mites. High rates of isolation of *Malassezia* were recorded in domestic carnivora affected by otitis externa, as reported in previous studies (15). *M. pachydermatis* was identified in pure culture in all samples resulted positive at cytological examination, suggesting the role of this fungal species in the aetiology of otitis externa in dogs and cats (16).

Among the perpetuating factors, bacteria and yeasts are believed to be less frequent in feline than in canine otitis cases (5). However, in the present study the occurrence of *M. pachydermatis* infection was slightly higher in cats (58.2%) than in dogs (52.7%). As found by other authors (17, 18, 19), staphylococci were the most commonly isolated bacteria. In addition, all bacterial species/genera here isolated were already reported in dogs and cats with otitis externa (3, 18, 20).

The presence of *O. cynotis*, a mite considered a primary cause of otitis externa, was significantly higher in cats than in dogs (66.1% and 5.9%, respectively), as reported in literature (2). According to some authors (5, 7), when bacteria or *Malassezia* yeast are present in cats with otitis externa, systemic medications should be considered, even if the middle ear is not involved.

It is important to note that bacteria or yeasts associated with cases of otitis externa are only opportunists and are not primary pathogens (2, 3, 5); in fact they are normally present in low numbers in the external ear canal. When a primary disease damages this anatomic component of the ear, the normal microflora can proliferate and exacerbate or perpetuate inflammatory reactions (2). Although information about other primary factors as allergies and endocrinopathies was not available, in cats *O. cynotis* infection was probably one of the main factors for the *Malassezia* overgrowth, especially considering the high prevalence (about 70%) of cats colonised by *O. cynotis*. However, from the analysis of data obtained in this study, there was no significant association between *Malassezia* and the other primary and/or perpetuating pathogens which could lead to otitis externa in cats and in dogs. Nevertheless, expecially in case of bacteria, the existence of a possible correlation with *Malassezia* could have been masked by the relatively low number of examined animals and further studies are needed. Statistical analysis

confirmed that ear conformation (P < 0.05) is an important predisposing feature in pathogenesis of *Malassezia* otitis in dogs (2, 3, 6, 21). Other anatomical and conformational factors, such as seborrhea, high moisture levels in ear canals, hypoplastic and stenotic ear canals and a high density of hair in ear canals, are known to be predisposing factors to otitis externa (2, 3, 7, 9). However, in this retrospective study it was not possible to evaluate these features because information about these data were lacking in examined animals. The influence of the season on onset of *Malassezia* infections in cats appeared also significant. This latter correlation has not been extensively investigated before; however, winter seems a predisposing season for ear *Malassezia* infections in this animal species (16).

In conclusion, in both dogs and cats, emphasis should be placed on establishing a diagnosis of otitis externa through physical, parasitological and cytological examinations, followed by culture and molecular diagnosis (1, 7, 9, 22), allowing a more correct etiological diagnosis and a specific treatment schedule, and to overcome recurrences due to an improper management.

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MALASEZIJA, PRŠICE IN BAKTERIJE V UŠESNEM KANALU PRI PSIH IN MAČKAH OB VNETJU ZUNANJEGA SLUHOVODA

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Povzetek: S citološko, gojitveno in mikroskopsko analizo ušesnega masla smo določili pojavnost kvasovk iz rodu *Malassezia*, pršic in bakterij pri 115 mačkah in 203 psih ob vnetju zunanjega sluhovoda. Identifikacija malasezije je bila opravljena tudi z metodo PCR. Analizo na prisotnost malasezije smo opravili pri vseh bolnikih, na prisotnost pršic pa pri vseh mačkah in 101 psu. Bakteriološka analiza je bila opravljena pri 16 mačkah in 60 psih. Analizirali smo povezavo med pojavnostjo malasezije in ostalimi patogeni ter korelacijo med pojavnostjo malasezije in letnim časom, spolom ter obliko ušesa. *Malassezio pachydermatis* smo izolirali iz ušesnega masla 58,2 % mačk in 52,7 % psov, *Otodectes cynotis* pri 66,1 % mačkah in 5,9 % psov. Bakterije smo odkrili pri 18,7 % mačk in 36,7 % psov. Med bakterijami smo ugotovili vrste *Staphylococcus pseudointermedius*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Streptococcus canis*, *Escherichia coli* in *Bacillus sp*. Vrsta *M. pachydermatis* je bila izolirana v čisti kulturi pri 28,3 % mačk in 87,1 % psov, skupaj z O. cynotis pri 70,1 % mačk in 5 % psov, skupaj z bakterijami pa pri 1,5 % mačk in 23,3 % psov. Pri 1 psu in 1 mački smo določili mešano okužbo z *M. pachydermitis*, bakterijami (*S. pseudointermedius*) in pršicami. Naši rezultati tudi kažejo, da je pri psih oblika ušesa pomemben preddispozicijski dejavnik za pojavnost vnetja ušesa z malasezije. Pri mačkah pa se malasezije pojavlja sezonsko.

Ključne besede: *Malassezia pachydermitis*;*Otodectes cynotis*;bakterije; *otitis externa*; pes; mačka

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Original Scientific Article

CHANGES IN BLOOD ANTIOXIDANT, BIOCHEMICAL AND HAEMATOLOGICAL PARAMETERS IN POLICE HORSES ON DUTY

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Summary: The objective of this study was to test the hypothesis that routine patrolling by police horses induces physiological changes of haematological and biochemical parameters, but not exercise-induced oxidative stress. Therefore, the activities of whole blood antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), activities of serum muscle enzymes, aspartate aminotransferase (AST) and creatine kinase (CK), as well as haematological and other biochemical parameters in police horses on duty were investigated. Correlations between antioxidant and muscle enzymes were also determined. Fourteen healthy, male, warm-blooded police horses were included in this study. Horses were transported to their place of duty where they patrolled for six hours at a walking pace, which was regarded as moderate physical activity. Blood samples for determination of SOD, GSH-Px, AST, CK, haematological and biochemical parameters were collected in the horses' stalls (basal values), after their transportation to the place of duty, immediately after duty, after transportation back to their stalls and after a 24-hour rest. No significant changes in the activities of antioxidant enzymes among different sampling times ocurred. Correlations between antioxidant and serum muscle enzymes activities were not significant at any of the sampling times. However, significantly higher ($p < 0.05$) AST and CK activities were found immediately after duty in comparison to basal values, which is probably the result of leakage from intact muscle fibres resulting from muscular activity, rather than a consequence of oxidative stress-induced muscle damage. Fluctuations of haematological and biochemical parameters reflect horses' physiological response to physical activity when on patrol. In conclusion, police horses did not develop exercise-induced oxidative stress while being subjected to moderate physical activity on duty. They appeared to be in good physical condition and fit for this type of duty. Moreover, our results indicate that the investigated horses could be used for patrolling for longer periods of time.

Key words: police horses; exercise-induced oxidative stress; antioxidant enzymes; serum muscle enzymes.

Introduction

Physical exercise has been shown to induce tissue damage via the oxidation of cellular components, such as membrane lipids, proteins and DNA (1,2). During exercise and rest, a number of potential intracellular sources of reactive oxygen species (ROS) generation exists in the skeletal muscles and heart. They are derived from the mitochondrial transport chain, xanthine oxidase catalysed reactions, NAD(P)H oxidase enzymes from the sarcoplasmic reticulum and plasma membrane redox systems. Superoxide anion is the main ROS in the muscle cell produced by either an incomplete reduction of oxygen in the electron transport chain or as a specific product of different enzymatic systems (3). Increased production of ROS may lead to exercise-induced oxidative stress (1,2,4). However, endogenous enzymatic and non-enzymatic antioxidants have demonstrated great adaptability in response to acute and chronic exercise (4,5).

Exercise-induced oxidative stress is believed to contribute to muscle fatigue and muscle fibre damage, which can lead to exercise intolerance

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and poor performance (5,6). Horses are unique athletes within the animal kingdom. Their survival in the wild depends partly on their capacity to provide explosive effort when necessary, in order to escape predators. Consequently, during heavy exercise a horse has a unique ability to almost instantaneously increase its oxygen uptake by factors of more than 60, which leads to increased mitochondrial production of ROS (1,4,7). The latter could favour membrane lipid peroxidation of muscle cells and thereby decrease their membrane integrity, which could lead to tissue damage and muscle enzyme leakage (1,8). Associations between exercise-induced oxidative stress and muscle enzyme leakage have already been confirmed in sport horses (9,10,11).

Prolonged low-medium intensity exercise has been reported to induce oxidative stress in humans (5,12). In exercising horses, numerous studies have shown that exercise-induced oxidant/antioxidant changes vary with regard to the type of exercise (racing, standardised treadmill exercises, standardised race track exercises, endurance) and markers assessed in blood, although it is generally agreed that physical exercise does induce significant alterations in the circulatory oxidant/antioxidant balance. However, some controversy exists in terms of poorly reproducible and even contradictory results, which suggests that experimental design, the horses' fitness, the analytical approach and environmental factors strongly influence the study results (8,9,13,14,15) Physical exercise not only induces oxidative stress but can also modify the animal's physiological metabolism, leading to changes of physiological, haematological and biochemical parameters (7,16,17,18,19,20). In horses, all the studies that have investigated the impact of physical exercise on the oxidant/antioxidant balance and physical, haematological and biochemical parameters were conducted in sport horses, but none in police horses, which are daily subjected to different kinds of physical activity when on duty. The objective of this study was to test the hypothesis that routine patrolling of police horses induces physiological changes of haematological and biochemical parameters, but not exercise-induce oxidative stress. Therefore, we determined the activities of whole blood antioxidants, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), activities of serum muscle enzymes, aspartate aminotransferase (AST) and creatine kinase (CK), as well as haematological and selected biochemical parameters in police horses on duty (patrolling). In addition, correlations between whole blood antioxidant and serum muscle enzymes, whose increased activities may indicate muscle cell leakage induced by exercise-induced oxidative stress, were determined.

Material and methods

Horses

The study was conducted on fourteen healthy warm-blooded police horses, geldings, which were from five to seventeen years old (10.88 ± 0.87 years; n=14). They were transported for 80 kilometres in a double-horse trailer, with an average speed of 40 km/h, to the place of duty where they patrolled for the next 6 hours (from 10 a.m. to 4 p.m.). While patrolling, horses were ridden at walk through hilly woods, which was regarded as moderate physical activity. Horses were resting from 12 p.m. to 12:30 p.m. when they were provided food and water. The height difference that police horses had to overcome while patrolling was 274 m and approximate distance travelled was 35 km. After patrolling, they were transported back to their stalls. The transportation took approximately 2 hours in one direction. The horses were given water *ad libitum* and fed three times on the day they were on duty. At 5:30 a.m. and after transportation back at their stalls horses were given hay, oats and Cavalor Essential briquettes (Cavalor, Drongen, Belgium), at 12:00 p.m. horses were given only hay. The horses investigated were on the same duty the week before the study presented was performed.

All the horses included in this study were healthy and in a normal physical condition throughout the study. They belonged to the same mounted police department and were, therefore, subjected to the same police training programme, diet, vaccination and anti-helmintic procedures. While off duty, investigated police horses were exercised daily according to the following official training programme, which was set by police horse trainer: gymnastics exercise in manège (1 hour/day; gallop, gait, trot, etc.) or conditional field work (one and a half hour/day). Horses were normally exercised six days per week; one day per week was a resting time. At least twice a month,

the investigated horses were subjected to indoor and outdoor police polygon training that included: dressage, resting, gymnastics exercise and horse controlling, resting and training with disturbing elements (vigorous noises, 'smoke' test, gun shoots, alarm and other loud sounds, etc.). Twice a year, the investigated horses were also subjected to police polygon training (outdoor) in durations of three and five days. The training included outdoor tracking, dressage, restraint training, training with disturbing elements and final exercises.

During the study, the horses were carefully monitored for signs of injury, such as muscular pain, stiffness or lameness.

Collection and preparation of blood samples

Blood samples for determination of whole blood antioxidant enzymes, haematological and biochemical parameters were collected by venepuncture of the left jugular vein in the stall prior to transportation (basal values), immediately (within 5 minutes) after transportation to the place of duty (postTr1), after duty (postD) and after transportation back to their stall (postTr2), as well as after a 24-hour rest (resting-stall).

Blood samples for the determination of serum cortisol and biochemical profile, with the exception of lactate and glucose, were collected into serum separator tubes (Vacuette; Greiner Bio-One, Kremsmunster, Austria) and were left still for 30 minutes to clot prior to centrifugation at 1300 g at 4°C for 10 minutes. The serum was then separated and aliquoted into two cryotubes. Serum samples for the determination of cortisol concentration were kept frozen at -20 ± 2°C until analysed within two months after sampling. Biochemical profiles were determined on the day of sampling.

Blood samples for plasma glucose and lactate determination were collected into tubes containing lithium iodoacetate and heparin (Vacuette; Greiner Bio-One, Kremsmuenster, Austria). Tubes were centrifuged at 1500 g for 15 minutes at 4°C. Plasma was separated and analysed on the day of sampling.

Tubes with K_3EDTA anticoagulant (Vacuette; Greiner Bio-One, Kremsmunster, Austria) were used for the collection of blood samples for the determination of haematological parameters. Blood samples containing EDTA were stored at room temperature (20–22 °C) and analysed within 10 hours after sampling.

Blood samples for determining GSH-Px and SOD activities were collected into tubes containing the anticoagulant lithium heparin (Vacuette; Greiner Bio-One, Kremsmunster, Austria) and frozen within 10 hours after sampling at -80°C until analysis. The samples were analysed within two months after blood sample collection.

Biochemical analyses and serum cortisol determination

Biochemical profiles included serum electrolytes (sodium (Na), potassium (K), chloride (Cl), magnesium (Mg), inorganic phosphate (iP), calcium (Ca)), creatinine, urea, total protein, albumin, alkaline phosphatase (AP), CK, alanine aminotransferase (ALT) and AST, as well as plasma glucose and lactate. Na, K and Cl concentrations were determined with an electrolyte analyser Ilyte Na/K/Cl (Instrumentation Laboratory, Lexington, MA, USA). Biochemical profiles, with the exception of Na, K and Cl, were determined with an automated biochemical analyser RX-Daytona (Randox, Crumlin, UK).

Serum cortisol concentrations were determined using a commercial enzyme immunoassay (Active Cortisol EIA, DPC, Los Angeles, USA). Absorbances of calibrators and samples were measured spectrophotometrically using a microplate reader Anthos (Anthos Labtech Instruments GmbH, Salzburg, Austria). Cortisol concentrations were calculated by the WinRes computer programe, which is a functional part of the reading system. All samples were analysed with a single run, with variability coefficients of 2.05 and 5.29 for low ($\bar{X}$ =116.75 nmol/l) and high ($\bar{X}$ = 694.42 nmol/l) values, respectively.

Haematological analyses

Haematological analyses were performed using a Technicon H*1 automated laser haematology analyser (Siemens, Munich, Germany) with species-specific software (H*1 Multi-Species V30 Software, Tarrytown, New York, USA). The complete blood count (CBC) included white blood cells (WBC), red blood cells (RBC), haemoglobin concentration (Hgb), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and platelets (PLT). The white cell

differential count (WCDC) represent a six-part differential: neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), eosinophils (EOS), basophils (BASO) and large unstained cells (LUC). WCDC values are represented as a percentage. The LUC category consists of a heterogeneous population of all large cells that fail to exhibit any peroxidase activity (atypical lymphocytes, immature granulocytes and blasts).

Measurement of GSH-Px activity

The activity of GSH-Px in whole blood haemolysates was determined spectrophotometrically with an automated biochemical analyser RX-Daytona (Randox Laboratories, Crumlin, UK) using the commercial Ransel kit (Randox Laboratories, Crumlin, UK), which is based on the method of Paglia and Valentine (21). The activity of GSH-Px was expressed as Units/g of haemoglobin (U/g Hgb).

Measurement of SOD activity

SOD activity in whole blood haemolysates was determined spectrophotometrically with an automated biochemical analyser RX Daytona (Randox Laboratories, Crumlin, UK), using a commercially available Ransod kit (Randox Laboratories, Crumlin, UK), which is based on the original method of McCord and Fridovich (22). Activity was expressed as U/g Hgb.

Statistical evaluation

Data was analysed using the SPSS computer program (SPSS 17.0 for Windows, Chicago, Illinois, USA). Results are expressed as means ± standard error of the mean (s.e.m.). Changes of measured parameters (significant difference) were assessed with repeated measures of ANOVA with the Bonferroni correction. Pearson's correlation coefficient analysis was performed to determine the correlation between the activities of whole blood antioxidants and serum muscle enzymes at each sampling time. A value of $p < 0.05$ was considered significant.

Results

No significant changes in the activity of antioxidant enzymes were observed among different sampling times, while the activity of serum muscle enzymes, AST and CK, changed significantly (Table 1). In comparison to basal values, the activity of AST was significantly higher at postTr1, postD and postTr2; while the activity of CK was significantly higher at postD sampling time. However, the activities of AST and CK returned to near their basal values after a 24-hour rest.

Whole blood antioxidants and serum muscle enzymes activities were not significantly correlated at any sampling times (data not shown).

Table 1: Whole blood antioxidant and serum muscle enzymes activities (mean ± s.e.m.) in police horses before and after duty

Measured parameter	Reference ranges	Basal values	PostTr1	PostD	PostTr2	Resting-stall
SOD (U/g Hgb)	1411.6±246.9[23] 1284.0±81.0[24]	1220.3±65.0^{a}	1247.8±62.8^{a}	1252.0±59.0^{a}	1223.2±66.6^{a}	1237.2±63.4^{a}
GSH-Px (U/g Hgb)	215.0±9.0[24]	177.4±6.7^{a}	175.9±5.5^{a}	177.1±5.1^{a}	180.2±8.0^{a}	177.4±6.3^{a}
AST (U/L)	160-412[24]	263.4±10.0^{a}	276.9±12.3^{b}	287.2±10.4^{b}	303.3±13.2^{c}	273.5±22.4^{abc}
CK (U/L)	60-330[24]	165.5±15.5^{a}	200.3±22.4^{ab}	223.7±21.3a^{b}	226.7±32.5^{b}	199.0±21.4^{ab}

^{a,b,c}mean values with different superscripts in the same row differ significantly ($p < 0.05$); SOD - superoxide dismutase; GSH-Px - glutathione peroxidase; AST - aspartate aminotransferase; CK - creatine kinase

Table 2: Serum cortisol and biochemical parameters (mean ± s.e.m.) in police horses before and after duty

Measured Parameters	Reference ranges	Basal values	PostTr1	PostD	PostTr2	Resting-stall
Cortisol (μg/dL)	3.0-13.0[25]	18.7±4.1[a]	20.6±4.0[a]	20.5±4.2[a]	16.6 ±4.3[ab]	10.1±3.2[b]
Lactate (mmol/L)	<1.0[26]	0.65±0.04	0.65±0.05	0.81±0.09	0.74±0.05	0.66 ±0.05
Urea (mmol/L)	3.9-9.6[25]	5.3± 0.2[a]	5.4±0.2[abc]	5.6±0.2[abc]	5.8±0.2[b]	5.0±0.2[ac]
Creatinine (μmol/L)	35.4-194.5[25]	133.3±4.2[ab]	133.6±2.8[ab]	141.9±4.2[a]	141.1±4.6[ab]	125.6±4.2[b]
Glucose (mmol/L)	3.44-7.43[25]	5.8±0.2	5.4±0.1	6.0±0.2	5.8±0.1	5.5±0.1
Total protein (g/L)	56-76[25]	66.9±1.3[a]	68.8±1.2[ab]	69.6±0.8[bc]	72.1±1.4[c]	69.3±1.1[bc]
Albumin (g/L)	26-41[25]	39.1±0.6[a]	40.1±0.5[abd]	41.4±0.5[bd]	43.3±0.7[c]	41.3±0.4[cd]
Na (mmol/L)	128-142[25]	139.5±0.5[acd]	137.8±0.4[bd]	139.3±0.7[d]	140.8±0.9[c]	138.8±0.4[bcd]
K (mmol/L)	2.9-4.6[25]	3.60±0.08[a]	4.99±0.21[b]	4.62±0.13[bd]	4.14±0.24[acd]	3.29±0.17[ca]
Cl (mmol/L)	98-109[25]	99.6±0.5[a]	99.6±0.4[ab]	100.4±0.6[ab]	101.7±0.6[b]	99.3±0.4[ab]
Ca (mmol/l)	2.55-3.35[25]	3.01±0.07	2.99±0.07	3.03±0.07	3.26±0.26	3.00±0.08
Mg (mmol/L)	0.58-0.94[25]	0.88±0.03[ab]	0.87±0.03[ab]	0.83±0.03[a]	0.83±0.03[ab]	0.91±0.03[b]
iP (mmol/L)	0.48-1.52[25]	0.94±0.07[ab]	0.98±0.07[ab]	0.83±0.04[a]	0.97±0.07[ab]	1.11±0.05[b]
AP (U/L)	102-2[57]27	108.5±7.2[ab]	107.1±6.6[a]	114.8±6.2[ab]	117.5±7.7[b]	118.1±8.8[ab]
ALT (U/L)	3.0-23.0[28]	18.2±1.4	17.9±0.9	20.2±1.0	20.1±0.8	19.8±1.6

[a,b,c,d]mean values with different superscripts in the same row differ significantly ($p < 0.05$); Na - sodium; K - potassium; Cl - chloride; Ca - calcium; Mg - magnesium; iP - inorganic phosphate; AP - alkaline phosphatase; ALT - alanine transferase

Table 3: Haematological parameters (mean ± s.e.m.) in police horses before and after duty

Measured parameters	Reference range[A]	Basal values	PostTr1	PostD	PostTr2	Resting-stall
WBC (x10^9/L)	5.40-12.00	6.39±0.35[a]	7.30±0.39[b]	7.83±0.44[b]	7.74±0.38[b]	7.00±0.32[b]
RBC (x10^{12}/L)	6.80-12.90	7.64±0.18[a]	8.08±0.13[ab]	8.41±0.21[ab]	8.18±0.18[ab]	8.13±0.14[b]
HCT (L/L)	0.32-0.53	0.35±0.01	0.38±0.01	0.39±0.01	0.38±0.01	0.37±0.01
Hgb (g/L)	110-180	136.8±4.0	143.9±2.1	149.6±2.8	145.4±2.1	142.2±2.3
MCV (fL)	34.0-55.0	46.0±0.8[a]	47.0±0.8[b]	46.9±0.8[b]	47.0±0.8[b]	46.4±0.7[ab]
MCH (pg)	12.3-19.0	17.8±0.3	17.8±0.2	25.2±7.2	17.8±0.3	17.8±0.3
MCHC (g/L)	340.0-410.0	387.9±4.0[a]	379.7±3.8[b]	381.1±4.1[b]	379.7±4.2[b]	384.9±2.9[ab]
PLT (x10^9)	50-350	145.8±7.9	138.9±5.6	142.7±4.9	145.6±7.1	144.9±7.4
NEUT (%)	40-75	58.0±1.6[ae]	65.1±1.6[b]	69.5±1.4[c]	71.7±1.1[cd]	59.4±2.2[abe]
LYMPH (%)	20-50	33.4±1.5[ae]	26.4±1.2[b]	24.5±1.0[bc]	22.6±0.9[d]	32.9±1.9[e]
MONO (%)	2-8	2.8±0.6	1.9±0.4	1.5±0.4	1.0±0.2	1.7±0.4
EOS (%)	0-5	1.3±0.3[ac]	0.9±0.3[ac]	0.5±0.3[b]	0.6±0.3[bd]	1.4±0.4[abd]
BASO (%)	0-2	0.30±0.02	0.40±0.08	0.30±0.04	0.30±0.06	0.30±0.03
LUC (%)	0-2	4.3±0.6	5.3±1.0	3.7±0.4	4.0±0.3	4.5±0.4

[a,b,c,d]mean values with different superscripts in the same row differ significantly ($p < 0.05$); [A]Reference ranges for horses (data from haematological analyser Technicon H*1); WBC - white blood cells; RBC - red blood cells; Hgb - haemoglobin concentration; HCT - haematocrit; MCV - mean corpuscular volume, MCH - mean corpuscular haemoglobin; MCHC - mean corpuscular haemoglobin concentration; PLT - platelets; NEUT - neutrophils; LYMPH - lymphocytes; MONO - monocytes; EOS - eosinophils; BASO - basophils; LUC - large unstained cells (atypical lymphocytes, immature granulocytes and blasts)

Serum cortisol and the biochemical parameters are presented in Table 2. With the exception of glucose, Ca, ALT and lactate, all other biochemical parameters changed significantly among different sampling times.

The concentration of serum cortisol did not change significantly immediately after duty or after both transports. However, the mean value was significantly lower at resting-stall than at basal, postTr1 and postD sampling times.

Compared to its basal value, a significantly higher urea concentration was determined at postTr2, but it decreased significantly after resting, reaching the basal value. The concentration of creatinine was higher at postD and postTr2 than at basal value, however the differences were not significant. After a 24-hour rest, the concentration of creatinine returned to near basal concentration. Concentrations of total serum protein and albumin were significantly higher at postD, postTr2 and resting-stall sampling times than at basal values.

No significant changes of Na and Cl concentrations were observed immediately after duty when compared to their basal values. However, the concentration of Na was significantly lower at postTr1 and the concentration of Cl significantly higher at postTr2 sampling time in comparison to respective basal values. There were some significant changes in the concentration of these two electrolytes during other sampling times. Potassium concentrations were significantly higher postTr1 and postD in comparison to basal concentration. After resting, Na, K and Cl concentrations were near their respective basal values. Concentrations of Mg and iP did not change significantly at any sampling times when compared to basal values, but their concentrations increased significantly from postD to resting-stall sampling time.

The activity of AP did not change significantly at any sampling times when compared to its basal value. However, the activity was significantly higher at postTr2 than at postTr1 sampling time.

Some of the haematological parameters, WBC, RBC, MCV, MCHC, NEUT, LYMPH and EOS changed significantlly among different sampling times (Table 3). In comparison to their basal values, significantly higher WBC values were determinated at postTr1, postD, postTr2 and resting-stall sampling times, and significantly higher RBC values at resting-stall sampling time. MCV and MCHC values were significantly higher postTr1, postD and postTr2 than their basal values.

Compared to basal values, significantly higher NEUT values and significantly lower LYMPH values were determinated at postTr1, postD and postTr2 sampling times. After a 24-hour rest, the values of NEUT and LYMPH returned to near basal values. Significantly lower EOS values were determinated at postD and postTr2 than at basal measurements.

Discussion

Determination of the changes of whole blood antioxidant and serum muscle enzyme activities, as well as haematological and biochemical parameters, provides valuable information about the physiological adjustments of police horses to stressful conditions that these horses experienced while being on duty.

At the beginning of the study, the basal values of all haematological and biochemical parameters were measured. With the exception of the serum cortisol concentration that slightly exceeded the upper value of its reference range (it will be discussed later), all parameters were within their reference ranges (25,26,27,28).

Antioxidant enzymes, SOD and GSH-Px, constitute the primary antioxidant defence system against ROS and are thus sensitive markers of oxidative stress (5,29). An association between exercise-induced oxidative stress and muscle enzyme leakage has already been confirmed in sport horses (9,10,11), but not in police horses on duty. The present study established no significant correlations between whole blood antioxidant and serum muscle enzyme activities. Significantly higher activities of serum AST and CK found in investigated horses immediately after duty in comparison to their basal values are probably the result of leakage from intact muscle fibres resulting from muscular activity (19), rather than a consequence of oxidative stress-induced muscle damage. The latter is also supported by moderate increases in AST and CK activities immediately after duty and a decline after a 24-hour rest. It has been suggested that sampling at least 24 hours after exercise may reveal the differences between those animals showing a normal physiological response to exercise and those with abnormal or pathological response (19). In comparison to its

basal value, AST activity increased significantly after both transports. The transport of animals is physically demanding and can result in muscle damage and consequently in an increased activity of serum muscle enzymes (30). Despite significant increases of CK and AST activities, the values remained within their reference ranges (25).

Growing evidence indicates that the antioxidant defence systems of mammalian tissues are capable of adaptation in response to acute and chronic exercise (4,5). However, in sport horses, significantly higher (9,10,13,15) or significantly lower activity (31) of blood antioxidant enzymes, either SOD and/or GSH-Px, have been reported post-exercise as a consequence of enhanced ROS production, either by up-regulation of enzyme activity or utilisation of the antioxidant enzymes to counter the ROS (4,5). In contrast, the results of some studies demonstrate no significant changes of antioxidant enzyme activities post-exercise in comparison with pre-exercise values (18,31). The inconsistency of results on blood SOD and GSH-Px activities could be a reflection of differences in exercise intensity, duration, type, or training (8). The present study demonstrated no significant changes of SOD and GSH-Px activities in police horses on duty. The lack of significant changes in antioxidant enzymes might be ascribed to the low intensity and short duration of physical activity of police horses on duty, as well as to the acclimatisation of investigated horses to this kind of physical activity since they have been on the same route many times and are daily subjected to exercise training programme. Activities of antioxidant enzymes determined in investigated horses were in general agreement with previously reported data at all sampling times (9,23,24).

During exercise, both the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis are activated to improve oxygen delivery to the working muscles. The activation of these systems is demonstrated by rapid increases in the circulating levels of adrenocorticotropin (ACTH), cortisol, adrenaline and noradenaline, allowing these hormones to be utilised in the evaluation of exercise-induced stress (32,33). Increased concentrations of cortisol in plasma or serum were reported only in sport horses after physical exercise (17,20,32,34,35). In the present study, the concentration of serum cortisol exceeded the upper value of the reference range (25) from the beginning of the study (basal value) to postTr2 sampling time, with resting-stall values being within the reference range. Higher than normal concentrations of serum cortisol were determined in investigated horses, which could have been due to the pre-duty excitement that horses experience in their stalls before being transported to the place of duty and later due to transport stress (36). The level of serum cortisol did not change significantly immediately after duty in comparison to basal values, but its concentration was significantly lower after a 24-hour rest than at all other sampling times. This result might be ascribed to the effect of training (37) since police horses are trained regularly on the same route.

As a response to exercise, the level of lactate found in blood is generally regarded as an indicator of fitness and degree of training, because it reflects the dependence on anaerobic metabolic pathways (33,38,39). Significantly increased concentrations of plasma lactate have been reported post-exercise in sport horses (16,20,34,40). In the present study, plasma lactate remained within the reference range (26) at all sampling times and did not differ significantly among sampling times. The lack of plasma lactate response to the physical activity of investigated horses is probably a consequence of the training (7,41) of these horses, and the fact that the anaerobic metabolism was not reached in these horses due to moderate type of physical activity. The results of lactate concentrations indicate that the police horses used in this study were in good physical condition.

Patrolling of police horses resulted in a significant increase of total serum protein, albumin and potassium concentrations at postD sampling time. Significantly increased albumin and total serum protein concentrations, determined at postD, as well as at postTr2 and resting-stall sampling times, most probably indicate dehydration (25,42). A significant increase in serum potassium concentration at postTr1 and postD sampling times might be ascribed to the movement of potassium from muscle cells during muscular contraction, which is reflected by an increase in blood potassium concentration (42). It has been noted that the degree of exercise needed to produce this effect is slight (20 to 50 m of walking) and that continued light exercise does not result in further increase in plasma potassium (17).

Exercise has variable effects on the haemogram depending on work intensity (19). In human beings and most other animal species, exercise

results in physiologic leukocytosis associated with mobilisation of marginated neutrophils to the circulating pool and moderate to marked neutrophilia. In horses, the mobilisation of leukocytes based on excitement and exercise is masked by the concomitant increase in erythrocytes and blood volume as a result of splenic contraction (19,33). In the present study, the WBC count was significantly higher, not only at postD, but also at all other sampling times when compared to basal values. However, leukocytosis was not determined at any of sampling times. Concomitant with significant increases in the leukocyte count, relative values of neutrophils were significantly higher and relative values of lymphocytes significantly lower at postD, postTr1 and postTr2 than at basal sampling time, which could be attributed to the mobilisation of marginated neutrophils (19,33). The alterations in the differential count in the present study were those of a typical stress pattern and similar to those reported by Snow et al. (1982) in horses during prolonged exercise (17). Despite significant changes, relative values of neutrophils and lymphocytes remained within their reference ranges (Technicon H*1).

Exercise generally results in the mobilisation of splenic erythrocytes and, therefore, increases the oxygen transport capacity. The extent of the haematocrit increase is a function of exercise intensity. However, it should be noted that part of this increase is attributable to exercise-induced fluid shifts from intravascular to interstitial space (19,33). Significant increases in RBC, haematocrit and HGB were reported after different kinds of exercise in sport horses (16,17,20). In the present study, RBC were significantly higher at resting-stall in comparison to basal values, although the highest mean values were determined immediately after duty. These results might be due to the high spread of RBC measurements at postD sampling time, thus resulting in the occurrence of a high standard error. In association with the increases in RBC were elevations of haematocrit and haemoglobin values. The highest values of these two parameters were determined immediately after duty. However, the increase was not significant at any of the sampling times when compared to basal values, which might be due to moderate intensity of physical activity of police horses on duty.

Dehydration or haemoconcentration are features of exercise and heat exhaustion in horses performing in hot dry climates and over long distances (typically endurance races) (43). Total serum protein and albumin concentrations, as well as haematocrit, are often used as indicators of dehydration (25,42,44). In the present study, significantly increased serum total protein and albumin concentrations, as well as increases in RBC and haematocrit, determined at sampling times after the basal measurements indicate the development of a progressive dehydration.

In conclusion, the police horses did not develop exercise-induced oxidative stress while being subjected to moderate physical activity on duty. Fluctuations of haematological and biochemical parameters reflect horses' physiological response to physical activity when on patrol. After a 24-hour rest most of the measured parameters return to their basal values. On the basis of our results, we can also conclude that police horses used in this study were in good physical condition and fit for this type of duty. Moreover, our results indicate that the investigated horses could be used for patrolling for longer periods of time.

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SPREMEMBE KRVNIH ANTIOKSIDANTNIH, HEMATOLOŠKIH IN BIOKEMIJSKIH PARAMETROV POLICIJSKIH KONJ PRI NJIHOVEM SLUŽBENEM DELU

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Povzetek: Namen raziskave je bil testirati hipotezo, da rutinsko patruljiranje policijskih konj povzroči fiziološke spremembe hematoloških in biokemijskih parametrov, ne pa oksidativnega stresa kot posledice zmerne fizične aktivnosti, kateri so bili konji podvrženi med patruljiranjem. S tem namenom smo v raziskavi ugotavljali aktivnosti antioksidantnih encimov, superoksidne dismutaze (SOD) in glutationske peroksidaze (GSH-Px) v polni krvi, mišičnih encimov, aspartat-aminotransferaze (AST) in kreatin-kinaze (CK) v serumu kakor tudi vrednosti hematoloških in ostalih biokemijskih parametrov pri policijskih konjih med njihovim službenim deluom. Hkrati smo določali razmerja med antioksidantnimi in mišičnimi encimi. V raziskavo smo vključili 14 zdravih toplokrvnih policijskih konj. Odpeljani so bili na mesto, od koder so nato pričeli 6-urno patruljiranje s hodom. Med patruljiranjem so bili podvrženi zmerni fizični aktivnosti. Vzorce za določanje SOD, GSH-Px, AST in CK ter hematoloških in biokemijskih parametrov smo odvzeli v boksih domačega hleva (bazalne vrednosti), po njihovem transportu na mestu opravljanja službenega dela, takoj po opravljanem delu, po transportu nazaj v bokse ter po 24-urnem počitku. Med različnimi odvzemi krvi nismo ugotovili značilnih sprememb v aktivnosti antioksidantnih encimov. Ugotovili pa smo, v primerjavi z bazalnimi vrednostmi, značilno ($p < 0{,}05$) višje aktivnosti AST in CK takoj po opravljenem delu, kar je verjetno prej posledica prepuščanja mišičnih encimov iz nepoškodovanih mišičnih vlaken in mišične aktivnosti kot pa oksidativnega stresa. Nihanja hematoloških in biokemijskih parametrov odražajo normalen fiziološki odgovor na zmerno fizično aktivnost policijskih konj pri svojem službenem delu. Ugotovimo lahko, da zmerna fizična aktivnost, kateri so bili podvrženi policijski konji med rutinskim patruljiranjem ni povzročila oksidativnega stresa in da so konji v dobri fizični pripravljenosti ter primerni za tovrstno službeno delo. Poleg tega naši rezultati kažejo, da bi lahko policijske konje, ki so bili vključeni v raziskavo, uporabili za daljši čas patruljiranja.

Ključne besede: policijski konji; oksidativni stres kot posledica vadbe; antioksidantni encimi; serumski mišični encimi

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Original Scientific Article

SEASONAL VARIATIONS OF FOUR HONEY BEE VIRUSES IN PUPAE, HIVE AND FORAGER BEES OF CARNIOLAN GRAY BEE *(APIS MELLIFERA CARNICA)*

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Summary: The seasonal dynamic of four honey bee viruses in 18 honey bee colonies from six selected apiaries were studied using a specific reverse transcriptase-polymerase chain reaction (RT-PCR) method. In each investigated colony, pupae, hive and forager bees were sampled and tested for acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV) and black queen cell virus (BQCV) once per month during a 14 month-period. In pupae samples, only BQCV and DWV were found in very low percentages, with no major differences throughout the investigating period. In hive bees ABPV, BQCV, CBPV and DWV were detected, but in lower percentages than in foraging bees where the number of all four viruses was the highest. The BQCV was discovered in 100% of foraging bees and in 94% of hive bees. For the other viruses, seasonal differences were observed in hive bees and foraging bees; however, the highest seasonal variations were detected for ABPV. This study suggests that horizontal transmission of ABPV, BQCV and DWV may occur through contacts between social groups in the direction from forager to hive bees.

Key words: honey bee viruses; RT-PCR; pupae; hive bees; forager bees

Introduction

During the last decade, losses in honey bee colonies were observed worldwide and also in Slovenia, which triggered a great interest in the research to find out the causative agent for these losses. Besides already well-known and established honey bee pathogens, the focus of recent research studies is also on honey bee viral infections (1, 2, 3). The most frequently detected bee viruses are acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV) and deformed wing virus (DWV) (2, 4, 5, 6, 7, 8, 9, 10, 11, 12). In nature, ABPV, BQCV and DWV infect larvae and pupae, as well as adult bees, while CBPV produces symptoms only in adult bees (2, 4). The diagnostics of honey bee virus infections is complicated because the infected honey bee colonies usually do not show clinical signs of the viral diseases (13). In addition, honey bee colonies can be infected with more than one virus at the same time (2, 5, 14, 15). In both the apparently healthy and the diseased bee colonies, multiple viral infections have been reported, suggesting that other pathogens and environmental factors can also play an important role in bee pathology (1, 5, 6, 7, 8, 16, 17). The prevalence and seasonal

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variations for 6 bee viruses in 36 apiaries were reported for samples collected in the pupae stage and the adult bee in the spring, summer and autumn of 2002 in France. During a 1-year period the percentage of viruses was lower among pupae samples in comparison to adult bee samples (2). Viruses can be transmitted horizontally and vertically. In a horizontal transmission, viruses are passed among individuals of the same generation, while vertical transmission occurs with infected queens (4, 9).

The presence of the Varroa (*Varroa destructor*) over the decades has had an important impact on the honey bee viral pathology (10, 11, 12, 14, 18, 19). The Varroa mite is considered as an important vector of the viruses within the colony, the apiary and among the apiaries across long distances (20). The transmission of DWV from the infected to the uninfected bees by the Varroa mite has been experimentally demonstrated and a positive relationship has been shown between Varroa infestation and morphological deformities, such as small body size, shortened abdomen and deformed wings, which has also resulted in the death of the heavily infested bees (20). Nevertheless, it is certain that honey bee colonies simultaneously infested with the Varroa mite and infected with viruses could be the reason for colony losses (15).

Nosemosis is the parasitic intestinal disease of adult bees caused by the protozoan *Nosema sp.*, which is present worldwide. This microsporidian pathogen has a significant impact on health and longevity of the individual honey bees as well as on the overall colony survival and productivity (21). Co-infection of adult bees with CBPV and *N. ceranae* using *per os* or *per cutis* virus inoculation showed increased replication ability of the virus as well as bee mortality (22). The average infectious dose is estimated to be from 20 to 90 spores per bee, but in the acute form of the disease, 30 to 50 million spores can be found in the midgut (23).

In Slovenia, breeding of the indigenous Carniolan gray bee (*Apis mellifera carnica*) is only allowed under the protection of the national law. The seasonal variation of viral infections is not known for different age categories in this honey bee race. In this survey, the presence of ABPV, BQCV, CBPV and DWV in samples of pupae, hive and forager bees from 18 colonies was observed monthly over a 14 month-period. Additionally, the number of spores of *Nosema sp.* and the fall of the Varroa mite were monitored for each colony during this period.

Materials and methods

Field work was carried out in 6 professional apiaries located in 3 different geographical regions in Slovenia (Gorenjska, Primorska and Dolenjska). Colonies were placed on the same location during the entire study and were treated once during the summer time with Checkmite (Bayer, Germany) against Varroa. In each apiary, 3 Carniolan gray bee (*Apis mellifera carnica*) colonies were randomly selected at the beginning of the study. Over the 6-month beekeeping period from April to September 2010 and 2 months from April to May 2011, each apiary was visited by a veterinarian specialist for bee diseases once a month. Each investigated hive was opened and honey bee colonies were checked to assess the clinical signs of the diseases. During visits to each investigated colony brood, hive and foraging bees were sampled for laboratory analysis. The brood was sampled in the pupae developing stage; the hive bees were those that were light grey and collected on the brood comb (nursing bees), while the foraging bees were gathered from the beehive entrance as they returned from the pasture with pollen in the baskets. In total, 419 pool samples of pupae (n=131), hive bees (n=144) and foraging bees (n=144) were collected; each sample consisted of 30 individuals. Samples were then stored below minus 60 °C until their use.

The fall of the Varroa mite was counted every month. For this purpose, screened bottom boards were placed in each hive in the beginning of May 2010 and May 2011; thus, for the first month of each sampling year, the data of infestation with the Varroa mite were missing. Every month, the accumulated debris on the screened bottom boards was removed. In total, 108 units of accumulated debris originating from 18 colonies were taken to the laboratory for a Varroa mite count.

In the laboratory, 419 pool samples were homogenised in 30 mL of RPMI (Gibco, UK) in ULTRA-TURRAX® DT-50 dispersing tubes (IKA, Germany). In each bee sample, the spores of *Nosema sp.* were counted with a standard counting chamber (Neubauer hemocytometer). After homogenisation, the suspensions of samples were centrifuged for 15 minutes at 2500 rpm, and supernatant was recovered. The total RNA was extracted with the QIAamp viral RNA mini kit (Qiagen, Germany), using 140 µL

of supernatant, according to the manufacturer's instructions. Individual RNA samples were tested for the presence of nucleic acids of ABPV, BQCV, CBPV and DWV, using the One-Step RT-PCR kit (Qiagen, Germany), as previously described (24). A reaction mixture without RNA served as the negative control, and a known positive sample of each of the 4 viruses as the positive control. The size of each PCR product was compared to the 100-bp DNA ladder (Fermentas, Germany), and the results for each RT-PCR reaction mix were interpreted as positive or negative, according to the expected size of the DNA fragment (24).

Results

Over the 14-month period of visits and observations of clinical signs, none of the 18 investigated honey bee colonies from 6 apiaries showed evident signs of the diseases. Generally, the lowest average number of 4 viruses for each month was detected in pupa samples, followed by hive bees while the highest average number of viruses was detected in forager bees (Figure 1). The BQCV was detected in 4 of 131 (3%) and DWV in 2 of 131 (2%) pupa samples, while all pupa samples were negative for ABPV and CBPV (Figure 2, Figure 3). In hive bee samples, BQCV was found with a high percent (varied from 89% to 100%) for all months, while DWV was detected between 6% and 44% of samples during the 14-month period (Figure 4). The ABPV was detected in April, May, June and August 2010 and in May 2011 with 22%, 17%, 17%, 28% and 89% positive samples, respectively. The CBPV was found only in 6% of hive bee samples in May 2011. The detection of positive results among hive bee samples revealed different percent for each of the 4 viruses, with the highest for BQCV (94%), followed by DWV (24%), ABPV (22%) and CBPV (1%) (Figure 3). In forager bees, BQCV was discovered in all samples (100%), but for DWV was lower and varied between 33% and 67% during the study period. The highest seasonal variation in the forager bees was observed for ABPV; 0% in April 2011, 6% in September 2010, between 39% and 50% from April to August 2010, with the highest percent of positive samples in June 2010 (83%) and May 2011 (100%). The detected variation for CBPV was from 0% (April, May and July 2010 and April 2011) to 28% positive samples (6% in June 2010 and May 2011, 11% in September 2010 and 28% in August 2010) (Figure 5). The comparison of all the results for 144 forager bee samples indicates that BQCV was detected in 100%, DWV in 50%, ABPV in 46% and CBPV in 6% of samples (Figure 3). Multiple virus infections were detected, with the highest number in forager bees for all months, followed by hive bees, while multiple infections were not detected in pupas (Figure 1). The peaks of the highest average number of multiple virus infections were detected in June 2010, August 2010 and May 2011 (Figure 1).

In 75 out of 108 collected samples (69,4%) of accumulated debris at least 1 Varroa mite was found (Table 1). The number of counted Varroa mites in 3 selected colonies within individual apiaries in the same month was similar. The highest numbers of Varroa mites were detected for all months in apiaries 1 and 3, followed by apiaries 2 and 5, with the highest numbers of Varroa mites counted in August and September 2010. In apiary 6, the highest numbers were counted in May and August 2010, while in apiary 4, the infestation was the lowest through the season, with 0 to 5 detected Varroa mites/per month (Table 1). The peak of Varroa mite infestation was detected in August 2010, with an average of 62 mites per colony. August 2010 was also the only month when all colonies were found infested with the Varroa mite (Table 1). The highest numbers of viruses and Varroa mites were detected in apiaries 1 and 3, while the lowest numbers were detected in apiaries 4 and 6, indicating strong correlation between Varroa mite infestation and number of detected viruses (data not shown).

Pupa samples were free of *Nosema sp.*, except 2 samples in May 2010 (0,2 and 0,4 million spores/bee) and 1 sample collected in May 2011 (1,8 million spores/bee). The comparison of average number of counted spores of *Nosema sp.* for 8 months showed the highest number detected in forager bees and a much lower number among hive bees (Figure 6). Both curves showed a similar dynamic; two peaks were recognised, first in April 2010 and second in September 2010. The average number of spores varied from 1.9 million spores per bee in June 2010 and May 2011 to 20.8 million spores per bee in April 2010 in the forager bee samples and from 0 spores per bee in June 2010 to 3.9 million spores per bee in September 2010 in the hive bee samples (Figure 6).

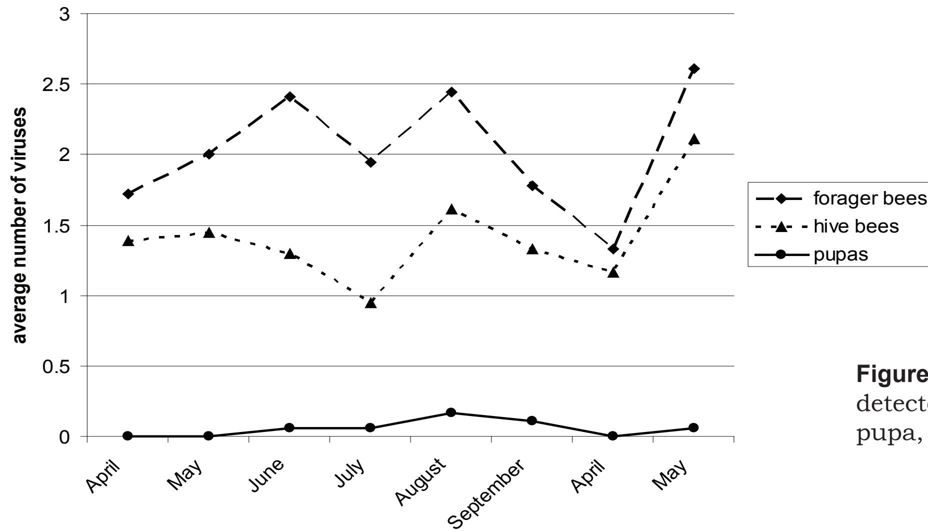

Figure 1: The average number of 4 viruses detected from April 2010 to May 2011 in pupa, hive and forager bee samples

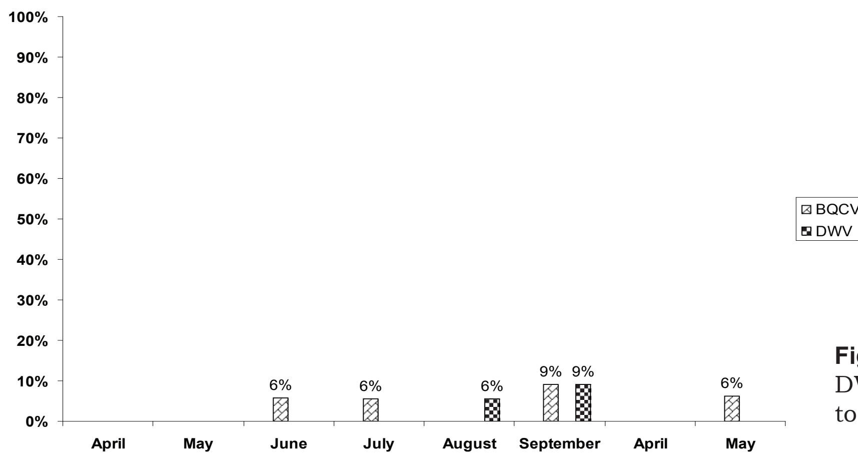

Figure 2: The percentage of BQCV and DWV detected in pupae from April 2010 to May 2011

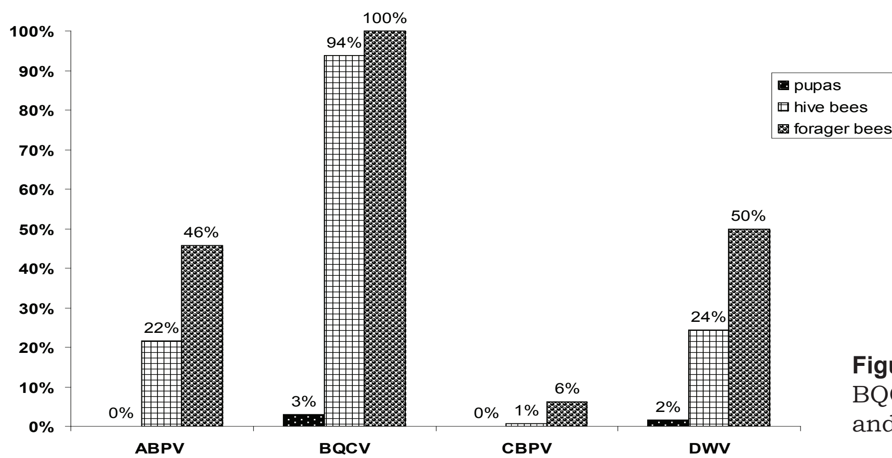

Figure 3: The percentage of ABPV, BQCV, CBPV and DWV in pupa, hive and forager bee samples

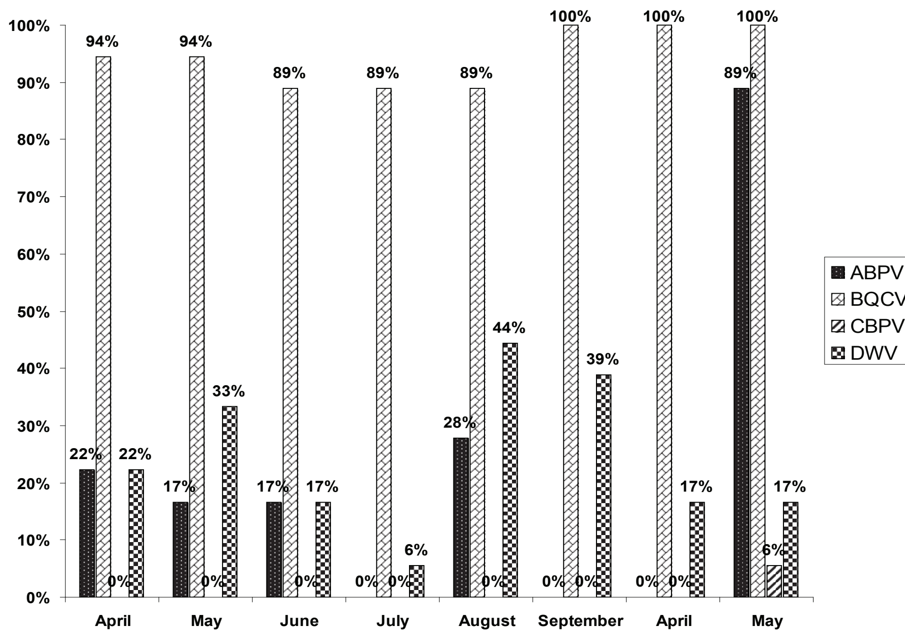

Figure 4: The percentage of ABPV, BQCV, CBPV and DWV detected in hive bee samples from April 2010 to May 2011

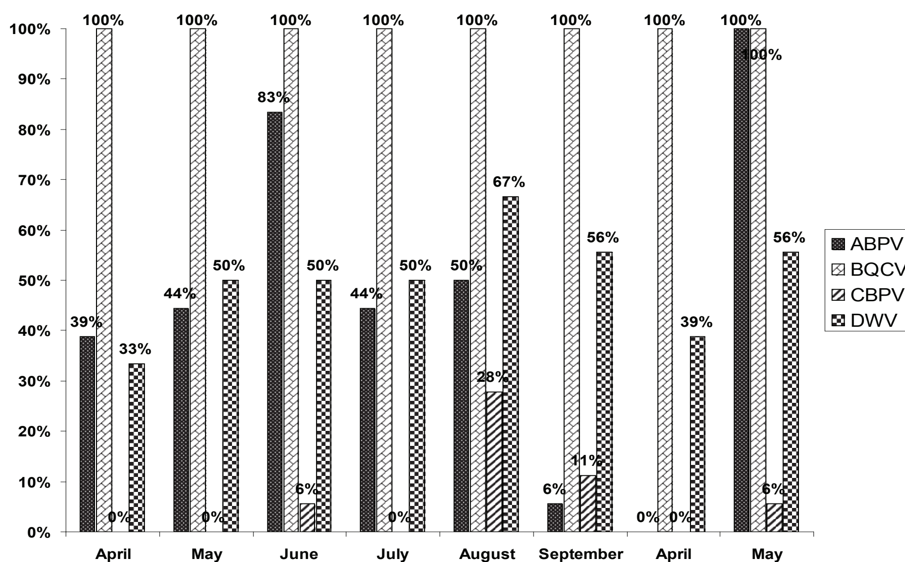

Figure 5: The percentage of ABPV, BQCV, CBPV and DWV detected in forager bee samples from April 2010 to May 2011

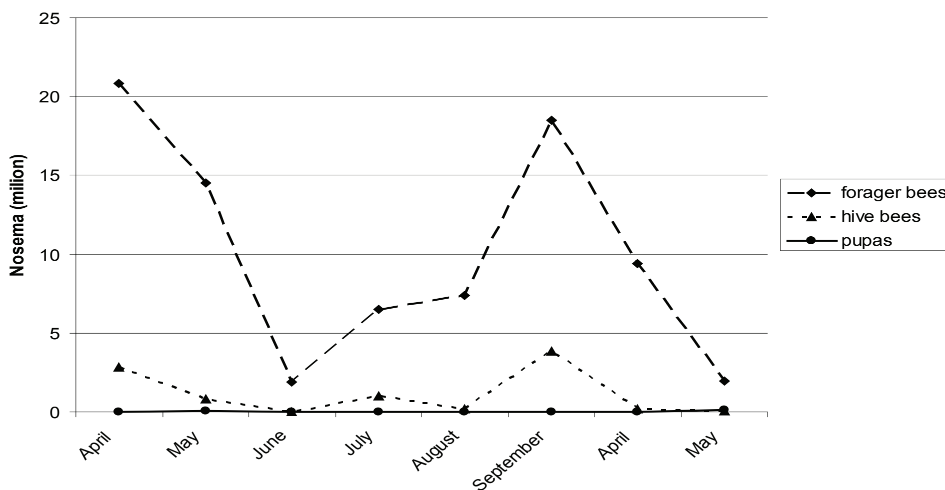

Figure 6: The average number of *Nosema sp.* (million/bee) from April 2010 to May 2011 in pupa, hive and forager bee samples

Table 1: Number of fallen Varroa mite in 18 investigated colonies detected from May 2010 to May 2011

		April	May	June	July	August	September	April	May
apiary 1	**colony 1**		4	11	42	73	21		5
	colony 2		3	49	15	45	35		3
	colony 3		17	23	200	124	30		7
apiary 2	**colony 1**		0	0	0	60	13		0
	colony 2		0	2	7	60	8		0
	colony 3		0	0	0	30	17		0
apiary 3	**colony 1**		20	52	140	200	100		3
	colony 2		21	15	33	100	100		2
	colony 3		18	29	45	300	25		5
apiary 4	**colony 1**		0	0	3	5	4		0
	colony 2		0	1	2	1	2		0
	colony 3		0	0	5	2	0		0
apiary 5	**colony 1**		0	1	1	28	15		0
	colony 2		0	0	0	14	15		0
	colony 3		0	0	4	33	0		0
apiary 6	**colony 1**		4	0	1	2	3		0
	colony 2		40	0	2	20	4		1
	colony 3		35	0	1	18	8		5

Discussion

In the last decade, several studies on the viruses in *Apis mellifera* from different countries have been reported, but there are only a few reports for seasonal variations of virus infections within colonies and apiaries. Viruses can infect all bee casts and developmental stages of the bees in the colony, including eggs, brood and adults. Most of the reports describe the virus prevalence for adult bees, but only limited or partial data are known about the seasonal virus prevalence for different age categories of the bees (2, 5, 15).

This study presents the prevalence of 4 honey bee viruses in pupae, hive bees and forager bees collected in 18 colonies from 6 apiaries over a 14-month period. To our best knowledge, this is the first study on the seasonal variation of 4 viruses in 3 different age categories of the bees, sampled monthly through the whole beekeeping season. In addition, the Varroa mite infection and *Nosema sp.* spores infestation in each investigated colony were screened monthly. The general prevalence of virus infections from the lowest to the highest was pupae < hive bees < forager bees. In our study, pupa samples were almost free of viruses; only in a very low number of samples BQCV and DWV was detected. The observed percent of positive samples detected in pupae was much lower, in comparison to the results of

a similar study published in 2002 in France (2). Although the prevalence of BQCV in hive bees and forager bees was almost 100% in all colonies, pupae were free of BQCV. A pattern similar to BQCV was observed for ABPV and DWV (Figure 3). A very possible interpretation of our results is that the majority of infections with these three viruses are the result of horizontal transmission, through contacts between social groups in the direction from forager to hive bees. The results from this study also suggest that the viral infections are less frequently transmitted vertically and few positive pupae samples are probably the result of the horizontal transmission from the infected hive bees, which were found positive in the same apiary, similar as described previously (4). Our hypothesis for possible seasonal variations of virus infections was proven, but not on the same level for different viruses. The percent of BQCV positive samples was stable through the months confirming that BQCV is present as a persistent infection without any clinical signs of infection in adult bees. Contrary, when comparing the results of ABPV in April and May 2010 to those in April and May 2011, some huge differences were observed. It is not clear why all the colonies were negative in April 2011, while this virus was frequently detected in season 2010 among forager bees in almost 50% of the colonies (Figure 5), but in May 2011, all colonies (100%) were detected as ABPV-positive, as completely opposed to one month ago (April 2011). This observation was also supported with the results of 89% ABPV-positive samples of hive bees in May 2011, confirming the results with the detection of high percent of positive forager bee samples and apiaries during last sampling, in May 2011. The detected seasonal variation for ABPV (0%-100%) in our study was much higher than observed in France (0%-25%) in 2002 (2) confirming high dynamic of this virus in short time period, which can be missed if period between two samplings is too long. In one of our previous studies (25), when the ABPV positive colonies had no clinical signs, low virulent ABPV strain was detected. This was confirmed already by the sequencing of part of the ORF 1 and ORF 2 regions of ABPV-positive samples with the identification of two genetic clusters of ABPV in Slovenia (25). All ABPV samples, except one which was collected from apiaries without clinical signs, belonged to the same genetic cluster 1, but for other apiaries infected with ABPV from cluster 2, clinical signs were described (25). The observed seasonal variations for DWV in forager bees ranged from 33% to 67%, confirming that this virus is constantly detected in all months of the season in almost half of the examined colonies. In our study low percent of pupae samples was discovered as DWV-positive (0%-9%), in comparison to the 95% DWV-positive pupae samples in DWV positive bee colonies in previous study (18). This observation confirmed that the comparison of the results between different studies is not easy and may be misleading if the purpose for both studies was different. In our study, CBPV was found mainly in forager bees, with a prevalence of 6% in June, 28% in August, 11% in September 2010 and 6% in May 2011 in hive and forager bees, while in other months, the virus was not detected. The direct comparison of the percent detected for ABPV, BQCV, CBPV and DWV in healthy forager bees from this study to the two previous publications of virus infections in clinically affected honey bees in Slovenia revealed a similar pattern for all 4 viruses (8, 24), but monthly variations for four viruses can be observed here for the first time for Carniolan gray bee. Our study also confirmed that multiple viral infections were detected through the whole season, with the highest frequency in forager bees, followed by hive bees, with some variations in the number of the detected viruses in different months, while only BQCV and DWV were found in a few pupae samples of the same colonies (Figures 1, 2, 4, 5). It is well known that a high number of the Varroa mite and *Nosema sp.* can weaken the bee's immune system, suppressing the expression of immune-related genes and increasing viral titers, both of which reduce honey bee life span and colony strength (26, 27). The numbers of viruses have been shown to be related to the degree of the Varroa mite infestation (2, 28, 29, 30, 31, 32). Worldwide and also in Slovenia, there are Varroa mite control strategies and programs to keep down the infestation at a low level. All bee colonies in our study were positive for the presence of Varroa mites at various levels. The monthly collected data for the fall of Varroa mites in 18 colonies revealed the major differences among the apiaries, while among the colonies of the same apiary, these differences were negligible (Table 1). The Varroa mite infestation was the highest in apiaries 1 and 3, but still far below the economic threshold and deviated from the results of the other four apiaries, where lower Varroa mite

infestation was detected (Table 1). The number of Varroa mites during each month showed the dynamic and classical relationship to the bee brood development, with increasing numbers of Varroa mites in spring and summer months (from May to July, peaking in August) and a drop in September (Table 1). When comparing the data to present the direct relationship between the number of Varroa mites and the number of viruses detected in each apiary, tendencies were confirmed; apiaries 1 and 3 (with the highest number of Varroa mites) also had the highest numbers of detected viruses, in comparison to those in apiaries 4 and 5 (data not shown). This is consistent with the previous observations that the Varroa mite is an important factor for spreading viruses (ABPV, DWV), but does not necessarily indicates if a colony will survive or collapse the following winter (15).

Monitoring the *Nosema sp.* infestation showed that the highest number was detected among forager bees, while the infestation in hive bees was significantly lower and the pupae were almost free of the pathogen, as expected.

Our study confirmed previous observations that BQCV is associated with *Nosema sp.*, although no symptoms in adult bees were observed if both pathogens were present (33). In the early spring, when infested long-living winter bees were still present in the colony, we observed a clear peak of the *Nosema sp.* infestation in April and a second peak later in September 2010, similar to another authors observation (23). Surprisingly, in April 2011, *Nosema sp.* infestation was much lower than that observed in April 2010, confirming that variations from season to season exist, as previously reported (34).

This study also provides further evidence for the long-lasting co-existence of multiple bee pathogens in apparently healthy Carniolan gray bee colonies, similar as observed before (2). So far neither of published results for viruses, together with the Varroa mite and *Nosema sp.*, were presented. Major differences in the virus prevalence among forager bees, hive bees and pupae collected from the same colonies/apiaries were observed. The results also showed that pupae were almost free of the pathogens, while hive bees were infected with viruses in a lower percentage than forager bees. It is reasonable to believe that these data reflect the horizontal transmission of viruses, as a result of the contacts among bees within the same colony. The Varroa mite and *Nosema sp.* were detected in every month of the sampling period with observed differences in prevalence between apiaries. Neither of these pathogens (the Varroa mite and *Nosema sp.*) was confirmed to be independently responsible for colony losses. Most probably, the results of multiple pathogen infections in different age categories of bees in a colony have pathological and/or synergistic effects on the individual honey bee immune system. Our research is presenting the complexity of the viral infections in Carniolan honey bees, which should be followed by further investigations.

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SEZONSKO POJAVLJANJE ŠTIRIH ČEBELJIH VIRUSOV V BUBAH, TER PANJSKIH IN PAŠNIH ČEBELAH KRANJSKE SIVKE (*APIS MELLIFERA CARNICA*)

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Povzetek: V študiji smo z metodo reverzne transkripcije in verižne reakcije s polimerazo (RT-PCR) ugotavljali prisotnost štirih različnih čebeljih virusov v 18 čebeljih družinah iz šestih izbranih čebelnjakov. V vsaki čebelji družini smo enkrat mesečno v obdobju 14 mesecev vzorčili bube ter panjske in pašne čebele in v odvzetih vzorcih dokazovali prisotnost virusa akutne paralize čebel (ABPV), virusa kronične paralize čebel (CBPV), virusa deformiranih kril (DWV) in virusa črnih matičnikov (BQCV). V vzorcih bub smo BQCV in DWV dokazali v zelo nizkem odstotku, brez pomembnejših razlik v spremljanem 14-mesečnem obdobju. Prisotnost vseh štirih virusov (ABPV, BQCV, CBPV in DWV) smo ugotovili pri panjskih čebelah, vendar v nižjih odstotkih kot pri pašnih čebelah, pri katerih smo ugotovili najvišje število virusov. Prisotnost BQCV smo ugotovili v 100 % vzorcev pašnih čebel in v 94 % vzorcev panjskih čebel. Pri panjskih in pašnih čebelah smo ugotavljali pomembna sezonska nihanja odstotkov pri ostalih treh virusih, najvišja nihanja pa smo ugotovili v prisotnosti ABPV. Ta študija zagotavlja tudi dokaze o možnem horizontalnem prenosu ABPV, BQCV in DWV od pašnih na panjske čebele med neposrednimi stiki med čebelami.

Ključne besede: virusi pri čebelah; RT-PCR; bube; panjske čebele; pašne čebele

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Original Scientific Article

MELAMINE-INDUCED NEPHROTOXICITY IN WEANED PIGLETS IN SERBIA

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Summary: Melamine is not approved for use in animal feed, although evidence of melamine poisoning in pigs has been found. Melamine in animal feed can induce nephrolithiasis, chronic kidney inflammation, bladder carcinoma and even death in animals. In September 2011, nine hundred 40–60-day-old piglets from a commercial finishing farm in Serbia developed anorexia, wasting, polydipsia and lethargy. At necropsy, the main macroscopic finding in ten necropsied piglets was observed on the kidneys. The kidneys were slightly enlarged and firm in consistency. The cortical surface was wrinkled, with a diffuse distribution of the yellow foci in the cortex and medulla. Microscopically, these yellow foci were accumulations of crystals located predominantly within the lumina of the dilated distal straight and convoluted tubules and collecting ducts. The crystals appeared variable and birefringent when viewed under polarized light. Two types of crystals were observed: the first type had several dark striations radiating from the eosinophilic round centre, while the second type consisted of pale green to brown irregular striated aggregates without an eosinophilic centre. In all cases, multifocally, there were moderate aggregates of lymphocytes, plasma cells, macrophages and multinucleated giant cells within the interstitium.
From the clinical signs, necropsy findings, histochemical and toxicological results, it was concluded that melamine-contaminated feed was the primary cause of nephrotoxicity and death in the observed piglets. To our knowledge, this is the first report of melamine poisoning in pigs in Serbia.

Key words: melamine; kidney; weaned piglets; nephrotoxicity

Introduction

Melamine (1,3,5-triazine-2,4,6-triamine) is a chemical product that used to be sporadically mixed into animal feeds to boost protein content; it gives a false elevation of the total protein content because the added melamine nitrogen is non-protein nitrogen. Melamine has no nutritive value and cannot be a substitute for proteins. Usually, melamine is used in the production of melamine resins, plastics, glues and inks (1, 2). Alkaline hydrolysis of melamine can produce three melamine derivatives: ammeline, ammelide, and cyanuric acid. Separately, melamine and cyanuric acid are relatively nontoxic in mammals (3). However, Brand (4) found that the addition of cyanuric acid to a diet containing melamine caused less toxicity in poultry than a diet contaminated with only melamine. Melamine and melamine derivatives cause poisoning in animals and humans (1, 3, 5-7). Melamine in animal feed can induce nephrolithiasis, chronic kidney inflammation, bladder carcinoma and even death in animals (1).

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Recent cases of nephrolithiasis and acute kidney injury among children in China have been linked to ingestion of milk-based infant formula contaminated with melamine. By November 2008, an estimated 300,000 victims had been reported, with six infants dying from kidney stones and other kidney damage, and a further 50,000 babies hospitalized (1, 7, 8). Although used mainly by the chemical industry, melamine was recently implicated in a foodborne outbreak of renal disease in domestic cats and dogs in the United States and Korea. In those cases, commercial pet food was adulterated with melamine (8-11). In swine, poisoning with melamine is characterized by anorexia, wasting, polydipsia, slight pallor and sometimes jaundice, lethargy and increased mortality. Sometimes there are no clinical symptoms (3, 5, 6). In one investigation, fish and pigs were fed a target dosage of melamine (400 mg/kg b.m.), cyanuric acid (400 mg/kg b.m.), or melamine and cyanuric acid (400 mg of each compound/kg b.m.) daily for three days. All animals fed the combination of melamine and cyanuric acid developed goldbrown renal crystals arranged in radial spheres (spherulites). Spectral analyses of crystals were consistent with melamine-cyanurate complex crystals. Although melamine and cyanuric acid appeared to have low toxicity when administered separately, they induced extensive renal crystal formation when administered concurrently (12). In one investigation, crystals were also found in one of the two pigs fed only melamine (200 mg/kg body mass per day) (13).

Melamine is not metabolized and is rapidly eliminated in the urine. One study, in which pigs were inoculated with melamine intravenously, demonstrated that melamine is rapidly eliminated by the kidney and probably not widely distributed to various tissues in the pig, and there should be no concerns about extensive binding to tissues that may be consumed by the public (14). However, methods for fast and on-site screening of melamine residue in animal tissues in order to eliminate the potential threat to human health have been developed (2).

In this paper, a case of accidental melamine poisoning of weaned piglets is described.

Material and Methods

Animals

In September 2011, nine hundred 40–60-day old piglets from a commercial finishing farm with 1500 sows, developed anorexia, wasting, polydipsia and lethargy. The piglets were fed with a complete feed mixture for weaned pigs. Feed intake gradually decreased by 20% to 40%. The morbidity rate gradually rose from 25% to 40% over a period of three weeks. Mortality began as early as three weeks after weaning and was 12% of the population of post-weaning piglets.

Pathological Examination

Necropsy was performed on the carcasses of ten clinically diseased weaned piglets, six males and four females, between 50 and 60 days old. For histopathological examination, samples of kidney tissue were taken, fixed in a 10% buffered formalin, routinely processed and embedded in paraffin blocks. Paraffin sections 4 µm thick were stained with haematoxylin and eosin (HE), Masson's trichrome and Periodic Acid Schiff (PAS), according to standard staining protocols, and the 72-hour Oil Red O (ORO72h) method described by Thompson et al. (10). All slides were viewed with light microscope Olympus BX51, and photos were taken with an Olympus Color View III camera. For the analysis of the observed crystals, slides were also viewed under polarized light.

Toxicological analysis

A milk replacer that was mixed in the feed for weaned piglets was suspected to be the source of the melamine; therefore, a sample was taken for toxicological analysis. The melamine content in pig milk replacer sample was determined with the HPLC/UV method. Extraction was carried out with trichloracetic acid and acetonitrile, followed by purification using cation-exchange SPE columns (Phenomenex Strata X-C). Melamine was eluted from the columns using 5% ammonium hydroxide in methanol. The eluate was evaporated to dryness in a nitrogen stream, and the dry residue was reconstituted in 1 mL of mobile phase, filtered through 0.22 µm nylon syringe filters, and 20 µl of extract was injected into HPLC system.

Chromatographic analysis was performed with a Waters Alliance 2695 Separation Module (Waters, Milford, USA) and UV-Vis with a Waters 2487 dual lambda absorbance detector. The wavelength was set at 240 nm. The HPLC column was reverse-phase C18 Phenomenex Gemini 150×4.6 mm, 5 μm (Phenomenex, USA). The mobile phase composition was 30% acetonitrile and 70% 2.5 mM SDS in 0.01 M of citric acid. Melamine was eluted using an isocratic program; the mobile phase flow was 0.6 mL/min. An analytical standard of melamine was purchased from Sigma-Aldrich (USA). Five-point calibration was performed at the beginning of each sample batch. Quality control samples were blank and fortified blank feed samples (3 mg/kg b.m.). Recovery of the analytical method was 75%.

Results

Pathology

In all examined weaned piglets, necropsy revealed dehydrated and emaciated carcasses. The skin was pale, and the hair was rough. Lung oedema with foamy fluid in the trachea was observed in 4 out of 10 carcasses. The main macroscopic finding was observed on the kidneys. In all piglets, the kidneys were slightly enlarged, firm in consistency, the cortical surface was wrinkled, with a diffuse distribution of the yellow foci in the cortex and medulla (Fig. 1A). The renal capsule could be easily removed from the kidney surface. On the cut surface, yellow foci that looked like parallel lines were mostly observed in the cortico-medullary junction and renal crest (Fig. 1B). In addition, the renal pelvis was dilated, and the renal cortex and medulla were atrophied.

Microscopically, these yellow foci in the kidneys were accumulations of crystals predominantly located within the lumina of dilated distal straight and convoluted tubules and collecting ducts. (Fig. 2A). Some of these crystals appeared to be in the walls and lumina of blood vessels, in addition to the lumina of renal tubules. In four cases, renal tubules were disrupted, and the crystals were located in the adjacent interstitium. Marked distal tubular necrosis and intratubular crystals were present in all cases. In three cases, evidence of proximal tubular injury was also observed. The crystals measured up to approximately 20 to 100 μm in diameter. Larger crystals were more common in the medulla. Crystals were variable in shape and birefringent when viewed under polarized light. Two types of crystals were observed. The first type had dark striations radiating from the centre with an eosinophilic round centre and concentric lamina, while the second type of crystals was pale green to brown with irregular striated coarse granular aggregates and without the eosinophilic centre (Fig. 2B). Multi-focally, there were moderate aggregates of lymphocytes, plasma cells, macrophages and multinucleated giant cells within the interstitium (Fig. 2C). This finding was observed in all 10 cases. Moderate interstitial fibrosis was present in six out of ten cases, and it was clearly seen in sections stained with Masson's trichrome (Fig. 2D). Interstitial fibrosis and non-suppurative infiltrates indicated a chronic inflammatory response. Round centres in the first type of crystals, which were eosinophilic in HE staining, were PAS positive (Fig. 2E). In all ten cases, ORO72h staining revealed different degrees of positive staining in the first type of crystals (Fig. 2F). As in the PAS staining, ORO72h staining was exclusively found in the round centres of the first type of crystals.

Toxicological analysis

Melamine concentration in the pig milk replacer sample determined by HPLC/UV was 5 mg/kg of dry milk replacer. The milk replacer was added to the feed ration at 20%; therefore, the calculated final concentration of melamine in the daily feed ration was 1 mg of melamine per kg of feed mixture.

Discussion

Although, melamine is not approved for usage in animal feed, there is evidence of melamine toxicity, or melamine and cyanuric acid and melamine and its derivatives toxicity in pigs (3, 5, 6). In this study, poisoning of weaned piglets occurred accidentally, after using melamine-contaminated feed. The main clinical symptoms in post-weaning piglets were anorexia, wasting, polydipsia and lethargy. Similar symptoms were observed by Lee et al. (6). After the removal of the suspected feed, no further cases of illness were observed.

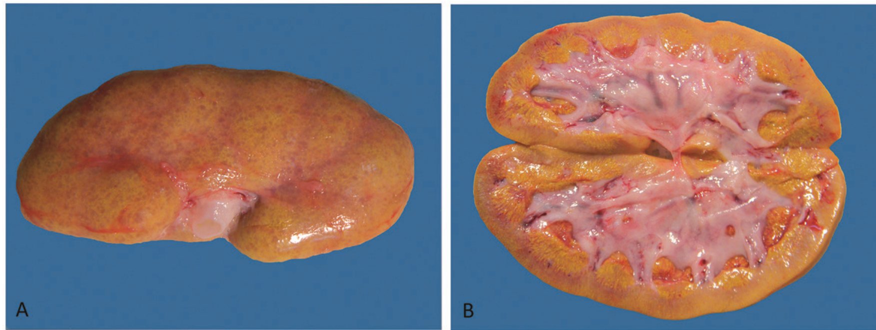

Figure 1: Gross lesions in kidneys after accidental melamine intoxication in weaned piglets. (A) Cortical surface of the kidney is yellowish and wrinkled. (B) Sagittal section of the kidney - diffuse distribution of the yellow foci in the cortex and medulla

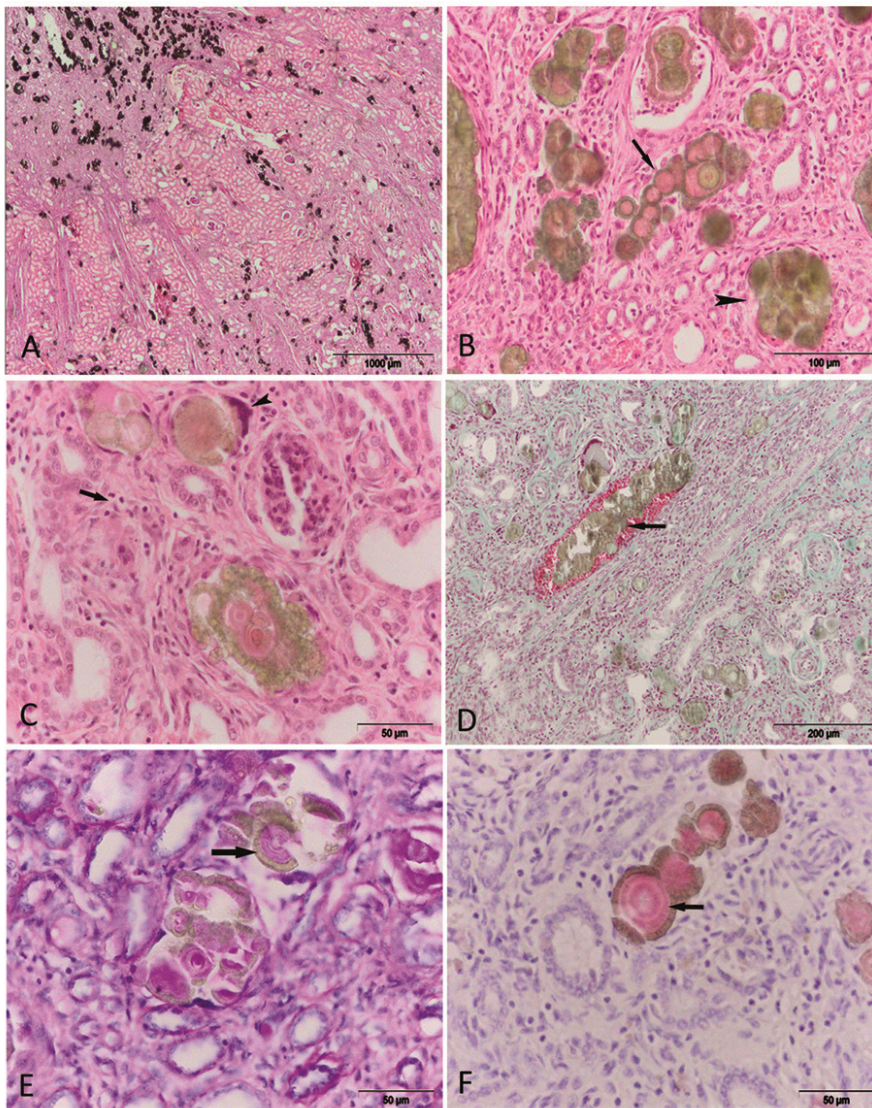

Figure 2: Microscopic lesions in accidental melamine intoxication in weaned piglets. (A) Accumulations of crystals predominantly within the lumina of dilated distal straight and convoluted tubules and collecting ducts. HE. (B) The first type crystals have several dark striations radiating from the centre with eosinophilic round centre (arrow), while the second type crystals are pale green to brown with irregular striated coarse granular aggregates and without eosinophilic centres (arrowhead). HE. (C) Moderate aggregates of lymphocytes, plasma cells, macrophages (arrow) and multinucleated giant cells around crystals within the interstitium (arrowhead). HE. (D) Moderate fibrosis – the renal interstitium is expanded by fibrous tissue (green). Crystal aggregates in blood vessel (arrow). Masson's trichrome. (E) PAS positive round centers in the first type crystals (arrow). PAS. (F) ORO72h positive round centres in the first type crystals (arrow). ORO72h.

The described macroscopic lesions in the kidneys are in accordance with findings described by Gonzales et al. (3) and Nilubol et al. (5). Wrinkled cortical surfaces, with yellow foci in the cortex and medulla can be characteristic lesions in melamine and/or cyanuric acid toxicosis in swine.

It is known that melamine is rapidly excreted in the urine and precipitated in the distal renal tubules (1), which is in accordance to our findings, showing accumulations of crystals predominantly within the lumina of dilated distal straight and convoluted tubules and collecting ducts. The first type crystals observed are similar to the crystals described by Thompson et al. (10) in three dogs with suspected pet food-induced nephrotoxicosis, and by Gonzales et al. (3) in swine, which were exposed to melamine and its derivatives. Similar crystals in melamine/cyanuric acid induced renal failure in dogs were described as "pinwheels" (11). Although ORO72h is primarily used for lipid staining, it is known from previous publications that prolonged staining with Oil Red O can be used for staining plastics (10), as we also found in our case in the first type crystals. Thompson et al. (10) found ORO72h positivity in melamine-containing crystals in dogs, and our results in swine are in accordance with their results. The first-type crystals were also positive to PAS staining. As the chemical structure of melamine includes three amino groups, it was thought likely that it would stain with PAS method.

Although melamine and cyanuric acid appeared to have low toxicity when administered separately, they induced extensive renal crystal formation when administered at the same time (12). In one investigation, crystals were also found in one of the two pigs fed 200 mg/kg body mass per day melamine only (13). Brand (4) found that the addition of cyanuric acid to a diet containing melamine caused less toxicity in poultry than a diet contaminated with only melamine. In our case, melamine was detected in the milk replacer, which was a component of feed for post-weaning piglets. It is possible that the melamine *per se* induced described pathomorphological changes, but the influence of other melamine derivatives cannot be excluded.

In this case, the diagnosis of nephrotoxicosis was based on the presence of crystals with characteristic light microscopic and histochemical properties, predominantly in the distal tubules and collecting ducts and subsequent demonstration of melamine in the milk replacer that was added to the complete feed. From the clinical signs, necropsy findings, and the histochemical and toxicological results, it could be concluded that melamine-contaminated feed was the most probable cause of nephrotoxicosis and death in the observed weaned piglets. Additionally, rather low concentrations (1 mg of melamine/kg of feed mixture) and a short period (3 weeks) of intake of melamine with feed may cause severe kidney damage, weight loss and death in weaned pigs. To our knowledge, this is the first report of poisoning due to melamine in weaned piglets in Serbia.

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NEFROTOKSIČNOST MELAMINA PRI ODSTAVLJENIH PUJSKIH V SRBIJI

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Povzetek: Živali sicer ni dovoljeno krmiti z melaminom, kljub temu pa obstajajo podatki o zastrupitvah s to snovjo. Če krma vsebuje melamin, lahko to povzroči nefrolitiazo, kronično vnetje ledvic, karcinom sečnika in celo pogin živali.
Septembra 2011 je v Srbiji na komercialni farmi za pitanje prašičev zbolelo 900 pujskov, starih 40 - 90 dni. Pri obolelih živalih so se pojavili anoreksija, hujšanje, polidipsija in letargija. Pri raztelesbi desetih pujskov so pri vseh ugotovili spremembe na ledvicah. Te so bile nekoliko povečane in čvrste konsistence, njihova površina je bila drobno nagubana, v skorji in sredici pa so bila difuzno razporejena rumena žarišča. Mikroskopsko so bila ta žarišča iz kristalov, nakopičenih v svetlini razširjenih premih, zavitih in zbirnih kanalčkov. Kristali so bili različne oblike in so lomili svetlobo pri opazovanju v polarizacijski svetlobi. Ugotovili smo dve vrsti kristalov: prvi tip je imel več temnih prog, ki so bile razporejene žarkasto okrog okroglega eozinofilnega središča, medtem ko je imel drugi tip bledo zelene do rjave nepravilno progaste agregate brez eozinofilnega središča. V vseh primerih smo v intersticiju ledvic ugotovili mnogožariščno razporejene skupine limfocitov, plazmatk, makrofagov in večjedrnih velikank.
Z analizo kliničnih znakov, ugotovitev pri raztelesbi, rezultatov histokemičnih in toksikoloških preiskav smo potrdili domnevo, da je bil glavni vzrok za nastanek sprememb v ledvicah in pogin pujskov krma, ki je bila kontaminirana z melaminom. Kolikor vemo, je to prvi opisani primer zastrupitve prašičev z melaminom v Srbiji.

Ključne besede: melamin; ledvica; odstavljeni pujski; nefrotoksičnost

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Original Scientific Article

COPPER, IRON, SELENIUM, ZINC AND MAGNESIUM CONCENTRATIONS IN OYSTERS (*Ostrea edulis*) FROM THE CROATIAN ADRIATIC COAST

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Summary: The aim of the study was to determine and compare the concentrations of essential elements (Cu, Fe, Se and Zn) and the macronutrient Mg in oysters (*Ostrea edulis*) from six different farming sites along the Croatian Adriatic coast. This is the first study to determine the accumulation of Cu, Fe, Mg and Se in oysters in Croatia. The concentrations of elements were determined in the ranges (mg kg^{-1}, wet weight): Cu 8.84–76.6, Fe 22.4–69.3, Se 1.66–4.88, Zn 129–737, Mg 607–1226. There were no significant differences in Fe, Mg and Se concentrations between sites. Significantly higher Zn and Cu levels were measured in oysters from Pag compared to those collected at localities in Mali Ston Bay. The results indicate an environmental influence on element levels in oysters. The estimated daily intakes (EDIs) calculated in oysters show a low contribution (0.008–2.52%) to the recommended dietary allowance (RDA) for these five elements. According to risk assessment results, it can be concluded that it is unlikely that the intake of Fe, Mg and Se through oysters would involve any risk for the average consumer. However, the high contribution of the EDI values to the provisional maximum tolerable daily intake (PMTDIs) obtained for Cu and especially for Zn suggests that oysters may pose a health risk to consumers.

Key words: Copper; Iron; Selenium; Zinc; Magnesium; Oysters; Croatia

Introduction

Ecological changes in water, as the result of human activity and pollution with heavy metals and other elements, has become a serious health concern due to the toxicity and accumulation of these elements by different aquatic organisms. Seafood products such as shellfish are known as accumulators of a number of pollutants, including the most studied biomagnifying substances, i.e. PCBs or methylmercury. They may accumulate essential and non-essential elements, which may exceed dietary recommendations (1). Accordingly, it is important to determine the health risk assessment and establish the benefits of seafood consumption.

For example, Zn-rich seafood may contribute to nutritional requirements for the antioxidant prevention of xenobiotic-induced oxidative stress and associated toxic hepatitis (2). Zinc has multiple biochemical functions and is present

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in a large number of proteins (3). Seafood can contribute to the attainment of the recommended intake levels of essential metals such as Cu and Zn (4). This may be important, particularly in developing countries with low socioeconomic development and nutritional deficiency of Zn. On the other hand, excessive Zn levels may cause acute and chronic toxicity and may lead to altered Fe function, reduced Cu status, reduced levels of high density lipid and reduced immune function (5). Acute Cu toxicity can cause nausea, vomiting, abdominal pain, headache, lethargy, diarrhoea, tachycardia, respiratory difficulties, haemolytic anaemia, gastrointestinal bleeding, liver and kidney failure and death. Chronic Cu toxicity can result in liver disease and severe neurological defects (6).

It is known that the oysters are an excellent source of copper (Cu), iron (Fe), selenium (Se) and zinc (Zn). Recently, the *Second French Total Diet Study* (7) indicated that the oysters species in the food group of "shellfish" are the group with the highest content of essential trace elements Cu, Zn and Se. Iron plays a major role as an oxygen carrier in haemoglobin in blood, or myoglobin in muscle, and it is also required for many metabolic processes (8). Selenium has roles in several critical metabolic pathways and in the immune and endocrine systems, and acts as an antioxidant showing enzymatic redox activity through essential enzymes as glutathione peroxidase (9). Magnesium (Mg) is required for more than 300 biochemical reactions in the body, including maintenance of normal muscle and nerve function and heart rhythm (10).

Although fish and seafood can contribute to achieving the recommended daily intake, they may also contribute to human exposure to environmental pollutants (11). Oysters and other marine species may serve as bioindicators for monitoring a variety of contaminants in the ecosystem. It has been confirmed that oysters originating from contaminated sites generally show significantly higher metal concentrations than controls (1).

The aims of this study were: I) to determine the levels of essential elements (Cu, Fe, Se and Zn) and the macronutrient Mg in oysters from six different farming sites along the Croatian Adriatic coast, II) to compare element levels between the different farming sites, III) to compare data of this study with previously reported data for these elements in oysters, and IV) to compare the estimated intakes with reference toxicological and nutritional values for each element.

Materials and methods

Sample collection

European flat oysters (*Ostrea edulis)* from six farming sites were collected during September and October 2011. Locations were sampled on different days. Ten oysters were collected at each sampling site, and three individual oysters (n=3, per site) were used for the analyses.

Oysters were collected from five oyster farms in the Mali Ston Bay and one farm from the island of Pag. Mali Ston Bay is situated between the mainland and the Peljesac Peninsula near the Neretva River delta on the southern Adriatic coast. Five oyster cultivation areas in Mali Ston Bay located near the village Mali Ston were: Brijesta, Mali Ston, Bistrina, Bjejevica and Sutvid (Figure 1).

The island of Pag is situated centrally on the eastern Adriatic coast. The oyster farm Stara Povljana is located in the closed Pag Bay near the main settlement on the island, the town of Pag. The town of Pag is known for sea salt production and is the island's main port.

Figure 1: Map of the sampling locations in Pag Island and Mali Ston Bay

Reagents and standards

All reagents were of analytical reagent grade, HNO_3 and H_2O_2 (Kemika, Croatia). Ultra high purity water processed through a purification system NIRO VV UV UF 20 (Nirosta d.o.o. Water Technologies, Osijek, Croatia) was used for all dilutions. Plastic and glassware were cleaned by soaking in diluted HNO_3 (1/9, v/v) and by subsequent rinsing with double deionised water and drying prior to use. Calibrations were prepared with Cu, Fe, Mg, Se and Zn standard solutions of 1 g L^{-1} (Perkin Elmer, USA). The stock solution was diluted in HNO_3 (0.5%).

Sample preparation

Shellfish meat samples (0.5 g) were digested with 4 mL of HNO_3 (65% v/v) and 2 mL of H_2O_2 (30% v/v) in a microwave oven. A high-pressure laboratory microwave oven (Multiwave 3000, Anton Paar, Germany) was employed to perform the acid digestion of samples. The digestion program began at a potency of 500 W, then ramped for 1 min, after which samples were held for 4 minutes. The second step at a potency of 1000 W (ramp 5 min) was held for 5 minutes. The third step at a potency of 1400 W (ramp 5 min) was held for 10 minutes. A blank digest was carried out in the same way.

Digested samples were diluted to a final volume of 50 mL with double deionised water. Concentrations of Cu, Fe, Mg, Se and Zn were determined on a wet weight basis as mg kg^{-1}. All samples were run in batches that included blanks, a standard calibration curve and two spiked specimens. The limits of detection (LODs, mg kg^{-1}) were determined as the concentration corresponding to three times the standard deviation of ten blanks in oyster samples: Cu 0.0021, Fe 0.005, Mg 0.008, Se 0.0018 and Zn 0.0015. The quality of data was checked by an analysis of the recovery rate using certified reference material: dogfish liver (DOLT-4, National Research Council, Canada). The reference material was treated and analysed under the same conditions as the samples. The results showed good accuracy with a recovery rate for tested elements (%): Cu 102.2, Fe 99.6, Mg 104.9, Se 105.2 and Zn 101.3.

Analysis of elements

An inductively coupled plasma optical emission spectrometer with axial and radial viewing plasma configuration (ICP-OES Model Optima 8000, Perkin-Elmer, USA) and an S10 autosampler

(Perkin Elmer, Waltham, Massachusetts, USA) was utilized. The instrumental operating conditions used are shown in Table 1.

Calculation of estimated daily intake

The estimated daily intake (EDI) was calculated by the equation (12):

EDI (mg kg^{-1} BW day^{-1}) = [(zinc concentration; mg kg^{-1}) per (meal size or daily intake of food; kg)] divided by [adult body weight (60 kg)]

In the calculation, the average consumption of shellfish in Croatia was set as 20 g day^{-1} per adult (13). The values of EDI were used to calculate contributions of each element to the reference nutritional (RDA, recommended dietary allowance, for females and males) and toxicological values (PMTDI, provisional maximum tolerable daily intake; TDI, tolerable daily intakes).

Statistical analysis

Statistical analysis was performed using the SPSS Statistics 17.0 software. Concentrations were expressed as mean ± standard deviation, median, minimum and maximum values. One-way analysis of variance was used to test for differences in elements levels in samples. Firstly, the normality of distribution was tested using Shapiro-Wilk normality test. Since the data was not normally distributed, a non-parametric test, Kruskal-Wallis ANOVA test, was used in defining the difference significance between the element concentrations from different locations. The difference between the groups was determined according to the test results. Thus, a multiple comparison by a Tukey HSD was also conducted to describe the differences and point out which groups differed significantly from others.

Results

The concentrations of five elements in the meat of oysters collected from six study sites are shown in Table 2.

Elements concentrations in oysters were measured in ranges (mg kg^{-1}): Cu 8.84 - 76.6; Fe 22.4 – 69.3; Mg 607 – 962; Se 1.66 – 4.88; Zn 129 – 737. There was a statistically significant difference between groups as determined by Kruskal-Wallis test for Zn levels on specific locations ($H(2) = 13.505$, $p = 0.019$). Thus, a Tukey post-hoc test was conducted and it revealed that the Zn levels of samples from Pag were significantly higher than those collected at locations in Mali Ston Bay, i.e. Brijesta, Mali Ston and Sutvid ($p<0.01$, all), including Bjejevica and Bistrina ($p<0.04$). Furthermore, Cu levels of the samples collected on Pag were statistically significantly higher than those from other locations ($p<0.021$, all).

There were no significant differences in Fe, Mg and Se concentrations between samples taken from different sites.

Table 3 shows the estimated daily intake (EDI) of elements based on the lowest and highest mean concentrations determined for each element. Also, the contribution of EDI values to the recommended dietary allowance (RDA), provisional maximum tolerable daily intake (PMTDIs) and tolerable daily intakes (TDI) were calculated for each element. The EDIs in oysters ranged from the lowest range of 0.0008 to 0.0011 mg day^{-1} for Se to the highest range of 0.242 to 0.314 mg day^{-1} calculated for Mg. The EDIs contributing to the RDA values were from the lowest percentage 0.008–0.024% for Cu to the highest contribution of 1.84–2.53% for Zn. Furthermore, contribution of the EDIs calculated for the lowest and highest mean of elements to PMTDIs values (%) were lowest for Se (0.0055–0.0076%) and highest for Zn (40.4–404%).

Discussion

To the extent of our knowledge, this is the first study to determine the accumulation of Cu, Fe, Mg and Se, alongside Zn, in oysters (*Ostrea edulis*) harvested in Croatia. In this study, Zn concentrations ranged from 129–737 mg kg^{-1}, with the highest mean concentration of 605 mg kg^{-1} measured at the Pag site. Among the localities in Mali Ston Bay, the highest level of 365 mg kg^{-1} was determined at Bjejevica. The results measured at the location Brijesta in Mali Ston Bay were much lower than those obtained in an earlier survey in Mali Ston Bay in oysters collected in February 2000 (> 600 mg kg^{-1}; 20). This may be explained by seasonal variations in element levels related to freshwater supply to the bay from the nearby Neretva River delta, which may be a key factor influencing element concentrations, particularly in winter and spring due to increased rainfall conditions (20). Increased freshwater inflow from

Table 1: Operating conditions for ICP-OES

Element / Parameter	Mg	Fe, Cu, Zn, Se
Plasma viewing mode	Radial	Axial
Read time	1-5 s	1-5 s
Measurement replicates	3	3
RF incident power	1000 W	1300 W
Plasma argon flow rate	8 L min^{-1}	15 L min^{-1}
Nebulizer argon flow rate	0.85 L min^{-1}	0.55 L min^{-1}
Auxiliary argon flow rate	0.2 L min^{-1}	0.2 L min^{-1}
Sample uptake rate	1.5 mL min^{-1}	1.5 mL min^{-1}
Inner diameter of the torch injector	2.0 mm	2.0 mm
Nebulizer type	Concentric glass (Meinhard)	Concentric glass (Meinhard)
Spray chamber type	Glass cyclonic spray chamber	Glass cyclonic spray chamber

Table 2: Concentrations of Cu, Fe, Mg, Se and Zn in oysters from six different locations from the southern and central Adriatic coast

Location	*N*	mg kg^{-1} wet weight					
		Statistics	Cu	Fe	Mg	Se	Zn
Brijesta (MS Bay)	3	Mean ± SD	26.4±5.89	54.2±3.63	828±139	3.15±1.50	196±37.1
		Minimum	22.9	52.1	668	2.28	175
		Maximum	33.2	58.4	909	4.88	239
Mali Ston (MS Bay)	3	Mean ± SD	29.2±14.4	44.5±11.8	880±84.3	2.74±1.16	168±34.0
		Minimum	19.6	33.5	793	1.66	129
		Maximum	45.7	57.1	962	3.97	189
Bistrina (MS Bay)	3	Mean ± SD	37.8±8.03	38.2±13.8	727±104	2.43±0.59	336±121606
		Minimum	28.5	22.4	607	2.05	216
		Maximum	42.9	47.4	795	3.12	417
Bjejevica (MS Bay)	3	Mean ± SD	31.4±6.44	52.1±13.0	804±120	2.49±0.69	365±72.0
		Minimum	26.9	44.4	676	1.82	289
		Maximum	38.8	67.1	914	3.20	431
Sutvid (MS Bay)	3	Mean ± SD	21.2±12.1	41.4±13.1	784±149	2.48±0.49	207±49.8
		Minimum	8.84	26.2	621	1.92	169
		Maximum	33.0	49.5	916	2.86	263
Pag (Pag Island)	3	Mean ± SD	65.1±13.6	44.2±22.1	1024±238	3.11±1.05	605±142
		Minimum	50.5	27.7	761	1.99	454
		Maximum	76.6	69.3	1226	4.08	737

Table 3: Estimation of daily intakes (EDIs) of elements based on the lowest and highest mean concentrations and contribution to reference nutritional (RDA) and toxicological (PMTDI) values.

Food	EDI[a] (mg kg^{-1} BW day^{-1})	Contribution of mean to RDA[b] (%)	Contribution of mean to PMTDI[c] or TDI[d] (%)
Cu			
Lowest mean	0.007	0.008 (F/M)	14.0 1.40
Highest mean	0.0217	0.024 (F/M)	43.4 4.34
Fe			
Lowest mean	0.0127	0.071 (F) 0.165 (M)	0.026
Highest mean	0.0181	0.10 (F) 0.23 (M)	0.038
Mg			
Lowest mean	0.242	0.078 (F) 0.061 (M)	-
Highest mean	0.341	0.11 (F) 0.085 (M)	-
Se			
Lowest mean	0.0008	1.45 (F/M)	0.0055
Highest mean	0.0011	2.00 (F/M)	0.0076
Zn			
Lowest mean	0.056	0.70 (F) 0.51 (M)	112 11.2
Highest mean	0.202	2.52 (F) 1.84 (M)	404 40.4

[a] EDI was calculated by the equation: [(element concentration; mg kg^{-1}) per (meal size or daily intake of food; kg)] divided by [adult body weight (60 kg)] (12). Meal size (g day^{-1} per adult): 20 g (13).
[b] RDA for female (F) and male (M): Cu 0.9 mg day^{-1} (F/M); Fe 18 mg day^{-1} (F), 8 mg day^{-1} (M); Mg 310 mg day^{-1} (F), 400 mg day^{-1} (M); Se 0.055 mg day^{-1} (F/M); Zn 8 mg day^{-1} (F), 11 mg day^{-1} (M) (14, 15).
[c] PMTDI (provisional maximum tolerable daily intake): Cu 0.05-0.5 mg day^{-1} BW day^{-1} (16); Fe 48 mg day^{-1} (17); Zn 0.3-1 mg day^{-1} BW day^{-1} (18).
[d] TDI (tolerable daily intake): Se 14.4 mg kg^{-1} (19).

various sources decreases salinity, which in turn significantly increases Zn levels in shellfish (21, 22). In comparing Zn levels with studies in other regions, Mali Ston Bay may be considered an area with low anthropogenic impact (1, 23).

The results obtained for all six sites were higher than Zn concentrations found in oysters *Ostrea edulis* from France (97 and 500 mg kg^{-1} (1); 181 mg kg^{-1} (7)). Previously, it was assumed that the Zn content in marine animals is dependent on the contamination of the water at the specific sampling sites, coastal region or open sea. The Zn content of molluscs and crustaceans is approximately four times higher than that of marine fish (3). The fact that oysters are strongly influenced by habitat contamination was previously confirmed by the determination of extremely high Zn concentrations of >900 mg kg^{-1}

(24). A good example of high variation regarding habitat contamination was found for the oyster species *Ostrea edulis* (British and French coast) and *Crassostrea gigas* (French Atlantic coast) at non-contaminated and contaminated locations, i.e. (mg kg^{-1}): *Ostrea edulis* 97 and 500; *Crassostrea gigas* 92 and 217, respectively (1). In the same study, maximal Zn values ranged from 214–800 mg kg^{-1} in the oyster *Saccostrea cucullata* from the rocky shores of Hong Kong (1). Extremely high Zn concentrations were found in oysters from Cleveland Bay (2080 mg g^{-1}) and from Orpheus Island (2547 mg kg^{-1}) in Australia (21). Previously, it was concluded that oysters accumulate high concentrations of Zn in detoxified granules (1). The present findings confirm high Zn concentrations in oysters and that oyster is a good alternative dietary source of Zn.

In the present study, the highest mean Cu content of 65.1 mg kg^{-1} was also determined in oysters from the Pag location (Table 2). The highest Zn and Cu levels at the site Stara Povljana on the island of Pag can be explained by the fact that this cultivation area is situated between the islands of Pag and Vir. In the summer months, this region has a high tourism load, which is not the case for the oyster cultivation area in Mali Ston Bay.

In French studies, the mean Cu concentrations measured in oysters were 12.9 and 11.7 mg kg^{-1} (7,11). These concentrations were 1.6–5.6 times lower than those found in the present study. Copper concentration differences in oysters from non-contaminated and contaminated locations from the coasts of Great Britain and France were previously determined (mg kg^{-1}): *Ostrea edulis* 6.4 and 78; *Crassostrea gigas* 13 and 58 (1).

There are few literature data providing other essential elements concentrations in oysters. In this study, Fe levels ranged from 22.4–69.3 mg kg^{-1} and the highest mean Fe level (54.2 mg kg^{-1}) was determined at the Brijesta location (Table 2). There were no significant differences in element contents between the locations from Pag Island and Mali Ston Bay. The results obtained were higher than those previously found (19.5 mg kg^{-1}; 11) but lower than recently reported levels in France (103 mg kg^{-1}, 25).

Magnesium concentrations were in the range from 621 to 1226 mg kg^{-1}. The highest mean Mg of 1024 mg kg^{-1} was determined at the Pag site, though there were no significant differences in Mg levels between the six sites. These results were similar to previously measured Mg concentrations of 1088 mg kg^{-1} in oysters from France (25). Selenium concentrations in this study were similar at all six sites and ranged from 1.66–4.88 mg kg^{-1}. The mean levels obtained were 5–17 times higher than those reported in studies from France (0.440 mg kg^{-1}, 11; 0.177 mg kg^{-1}, 7).

To evaluate the measured element levels in oysters from the nutritional and risk assessment perspectives, the estimated daily intake (EDI) was calculated and compared with recommended reference and toxicological values for each element. The sufficient recommended adequate intake of elements expressed as the recommended dietary allowance (RDA) for females and males are (mg day^{-1}): Cu 0.9; Fe 18 and 8; Mg 310 and 400; Se 0.055; Zn 8 and 11 (14, 15). As presented in Table 3, the EDIs calculated for the lowest and highest mean (chosen among means from all six sampling sites) for Cu, Fe and Mg represented very low percentages of the reference RDA values (ranging from 0.008% to 0.23% for a person weighting 60 kg). The EDIs calculated for Se and Zn showed a contribution to the RDA above 1.8% for highest measured mean values (%): Se 2.00; Zn 1.84 and 2.52.

For risk assessment purposes, the calculated EDIs of Cu, Fe, Mg, Se and Zn were compared with their toxicity reference values. Permissible human exposure as a result of the natural occurrence of elements in food were defined as the provisional maximum tolerable daily intake (PMTDIs) and tolerable daily intakes (TDI) and is used for risk assessment purposes associated with food consumption. Defined PMTDIs for elements were: Cu 0.05–0.5 mg kg^{-1} BW day^{-1} (16), Zn 0.3–1 mg kg^{-1} BW day^{-1} (18), Fe 48 mg kg^{-1} (17). For Se, the tolerable daily intake (TDI) is defined as 14.4 mg kg^{-1} (19). No PMTDI or TDI for Mg is given by World Health Organisation.

Contribution of the EDIs for Fe and Se calculated for the lowest and highest mean of elements to PMTDIs values were very low and below 0.05%. However, EDIs calculated at low and high mean level for Cu contribute to PMTDIs values in higher percent (%): low 1.40-14.0, high 4.43–43.4. The EDIs determined for Zn contribute to PMTDIs values at the lowest mean in the range 11.1–112% and at the highest mean in the range 40.4–404%. Therefore, the high values for Cu and Zn in comparison to the toxicity reference values suggest a risk from the consumption of oysters from all six sites.

To conclude, element variations in oysters were established between all collection sites. Significantly higher concentrations of Zn and Cu were determined in oysters from Pag compared to those from Mali Ston Bay, indicating an environmental impact on element levels. In order to investigate the environmental impact on the concentrations of elements, future studies should include element analysis in seawater and a survey of the monthly or seasonal variations in element concentrations at all six sites.

From the nutritional perspective, the estimated daily intake calculated for these five elements in oysters show a low contribution in accordance to the reference daily requirements for these elements. Following risk assessment results, it can be concluded that it is unlikely that the intake of Fe, Mg and Se through oysters would involve any risk for the average consumer. However, the intake of Cu and Zn through oysters may pose a health risk to consumers.

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KONCENTRACIJA BAKRA, ŽELEZA, SELENA, CINKA IN MAGNEZIJA V OSTRIGAH (*Ostrea edulis*) S HRVAŠKE JADRANSKE OBALE

N. Bilandžić, M. Sedak, M. Đokić, S. Zrnčić, D. Oraić, I. Varenina, B. Solomun Kolanović, Đ. Božić

Povzetek: Cilj raziskave je bil ugotoviti in primerjati koncentracije bistvenih elementov (Cu, Fe, Se in Zn) in makronutrienta Mg v ostrigah (*Ostrea edulis*) iz šestih različnih gojitvenih lokacij vzdolž hrvaške obale Jadranskega morja. To je prva študija o kopičenju Cu, Fe, Mg in Se v ostrigah na Hrvaškem. Koncentracije elementov so bile določene v mg/kg mokre teže in so se gibale v razponu: Cu 8,84 - 76,6, Fe 22,4 - 69,3, Se 1,66 - 4,88, Zn 129 - 737 in Mg 607 - 1226. Koncentracije Fe, Mg in Se se niso značilno razlikovale med različnimi gojitvenimi lokacijami, raven Zn in Cu pa je bila značilno višja v ostrigah s Paga v primerjavi z ostrigami iz zaliva Mali Ston. Naši rezultati kažejo pomemben okoljski vpliv na vsebnost ravni elementov v ostrigah. Ocenjeni dnevni vnos (EDIS) preiskovanih elementov z ostrigami predstavlja za človeka nizek prispevek (0,008 - 2,52 %) k priporočenemu dnevnemu vnosu (RDA). Glede na rezultate ocene tveganja je mogoče sklepati, da je malo verjetno, da vnos Fe, Mg in Se z ostrigami pomeni tveganje za povprečnega potrošnika. Nasprotno pa gre za visok delež vrednosti EDI pri najvišjem še sprejemljivem dnevnem vnosu (PMTDIs) za Cu in še posebej za Zn, kar kaže, da vnos Cu in Zn z ostrigami lahko predstavlja tveganje za zdravje potrošnikov.

Ključne besede: baker; železo; selen; cink; magnezij; ostrige; Hrvaška

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