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THE EFFECT OF PLACEBO-INHALER ON THE TRACHEAL EPITHELIUM IN RABBITS*

ÚČINEK PREPARÁTU PLACEBO-INHALER NA EPITEL TRACHEY KRÁLÍKŮ

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ABSTRACT: In experiment, the effect of two puffs of aerosol of Placebo-inhaler on the ultrastructure of the tracheal epithelium and on the composition of glycoconjugates of the goblet cells was studied. Using standard methods of electron microscopy, the ciliary border and the functional state of the goblet cells were evaluated quantitatively. The character of glycoconjugates was studied by means of methods of conventional and lectin histochemistry. 30 minutes after inhalation of propellants contained in the metered-dose inhaler, the ciliated cells revealed only slight signs of alteration reflected in a mild impairment of the ciliary border. 16% of goblet cells were stimulated to discharge mucus, 4% of them degenerated. Neutral glycoconjugates disappeared from the epithelium and insignificant decrease in acid sialylated glycoconjugates was demonstrated. Morphological signs of impaired self-cleaning ability of the tracheal epithelium were not encountered. The propellants contained in the metered-dose inhalers do not cause the damage to the airway epithelium due to the inhalation of bronchospasmolytic drugs.

airways; epithelium; Placebo-inhaler, ultrastructure; lectin histochemistry; glycoconjugates

ABSTRAKT: V experimentu jsme studovali účinek dvou vdechů preparátu Placebo-inhaler na ultrastrukturu epitelu trachey a na složení glykokonjugátů obsažených v pohárkových buňkách. Kvantitativně jsme hodnotili stupeň narušení řasinkového lemu nad epitelem a funkční stav pohárkových buněk. Pro studium složení glykokonjugátů jsme užili metody klasické a lektinové histochemie. 30 min po inhalaci propelentů obsažených v tlakových dávkovacích nádobkách jevily řasinkové buňky jen lehké známky patologické alterace, které se odrazily v mírném narušení řasinkového lemu. 16 % pohárkových buněk bylo stimulováno k vydávání sekretu, 4% z nich byly zcela vyprázdněné a degenerovaly. V epitelu došlo k nevýznamnému snížení množství kyselých sialovaných glykokonjugátů a neutrální glykokonjugáty zcela vymizely. Morfologické známky narušení samočisticí schopnosti epitelu jsme nezaznamenali. Propelenty obsažené v tlakových dávkovacích nádobkách nezpůsobují poškození epitelu dýchacích cest, které jsme pozorovali po aplikaci aerosolu bronchospasmolytických preparátů.

dýchací cesty; epitel; Placebo-inhaler; ultrastruktura; lektinová histochemie; glykokonjugáty

INTRODUCTION

In our previous studies (Konrádová et al., 1997, 1998; Vajner, 1998), the ultrastructure of the tracheal epithelium and the composition of glycoconjugates contained in the goblet cells' secretion were studied after inhalation of single therapeutic doses of two β_2 adrenergic agonists – salbutamol (Ventolin) and fenoterol hydrobromide (Berotec) – and a cholinergic antagonist – ipratropium bromide (Atrovent). Due to this treatment, the injury to the tracheal epithelium was moderate to severe. In the goblet cells' secretion, an absolute predominance of acid sulphated glycoconjugates was revealed. Using a light microscope, Spahr-Schopfer and Shorten with their fellow-workers described the adverse effect of salbutamol on the airway epithelium (Spahr-Schopfer et al., 1994; Shorten et al., 1995). As these authors suggested that the epithelial damage was most likely due to the Ventolin propellants, especially to the oleic acid contained in the metered-dose inhaler, we decided to study the effect of

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a single dose of Placebo-inhaler on the airway epithelium. This aerosol contains all the substances of Ventolin with the exception of salbutamol.

MATERIAL AND METHODS

In our experiments, 6 specific pathogen free New Zealand White rabbits (body weight 1 500–3 000 g, Charles River Deutschland, Sulzfeld, Germany) were used. The untreated controls represented the same groups of animals used in our previous studies (Konrádová et al., 1997, 1998; Vajner, 1998). The animals were treated with two puffs of Placebo-inhaler (Glaxo, Greenford, Great Britain). The metered-dose inhaler containing only propellants was connected with a long thin catheter that was inserted into the mouth of a rabbit under general anaesthesia and two puffs of aerosol were administered into its airways during spontaneous inspirium. The material for the electron microscopic and histochemical examination was collected 30 minutes post exposure. Tiny fragments of the tracheal mucous membrane were processed using standard methods for electron microscopy. The ciliary border and the functional state of the goblet cells were evaluated quantitatively as described in our previous paper (Konrádová et al., 1997, 1998). After administration of two puffs of Placebo-inhaler, 482 goblet cells and 2 150.5 μm^2 of ciliary border with 18 019 kinocilia were evaluated. Methods of conventional and lectin histochemistry described in our previous studies were used to study the character of glycoconjugates contained in the goblet cells' secretion (Vajner, 1998).

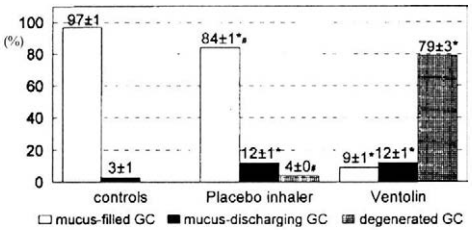
RESULTS

In control rabbits, the tracheae were lined with a ciliated pseudostratified columnar epithelium. The ciliated cells of standard ultrastructure were the most numerous

1. Glycoconjugates in the goblet cells (GC) in the tracheal epithelium of rabbis 30 minutes after inhalation of 2 puffs of propellants (Placebo-inhaler) and salbutamol (Ventolin)

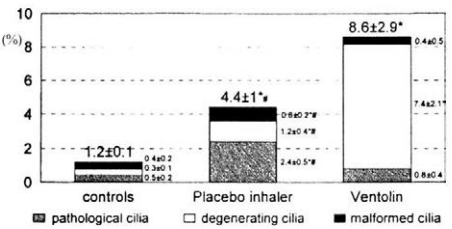
		Control rabbits	Propellants (Placebo-inhaler)	Salbutamol (Ventolin)
GC containing neutral GCC (PAS positive)	%	1.5 ± 2.4	0.0 ± 0.0	0.0 ± 0.0
GC containing acid GCC (AB pH 2.5 positive)	%	98.5 ± 2.4	100.0 ± 0.0	100.0 ± 0.0
GC containing sulphated acid GCC (AB pH 1 positive)	%	71.9 ± 6.4	74.2 ± 5.4	*100.0 ± 0.0
GC containing sialylated acid GCC (calculation)	%	26.6 ± 6.4	28.5 ± 5.4	*0.0 ± 0.0
GC containing sialylated acid GCC (MAA positive)	%	27.9 ± 9.6	24.4 ± 12.7	*0.0 ± 0.0
GC containing sialylated acid GCC (SNA positive)	%	2.3 ± 2.9	0.3 ± 0.5	0.0 ± 0.0
GC containing sialylated acid GCC (MAA+SNA positive)	%	30.2 ± 9.6	24.7 ± 13.2	*0.0 ± 0.0
GC containing sialylated acid GCC (TML positive)	%	26.6 ± 11.5	21.9 ± 7.4	*0.3 ± 0.5

Explanation:
n = 4 in controls, 3 in others, mean ± SD
values marked * differ significantly (α = 0.01) from controls, values connected by a line differ significantly (α = 0.01) from each other
GCC = glycoconjugates, PAS = periodic acid – Schiff reaction, AB = alcian blue
MAA = *Maackia amurensis* agglutinin, SNA = *Sambucus nigra* agglutinin, TML = *Tritrichomonas mobilensis* lectin



1. Functional state of goblet cells (GC) in the tracheal epithelium of rabbits 30 minutes after inhalation of 2 puffs of propellants (Placebo-inhaler) and salbutamol (Ventolin)

n = 3, mean ± SD
values marked * differ significantly (α = 0.01) from controls
values marked # differ significantly (α = 0,01) from corresponding values in the group of rabbits after inhalation of Ventolin



2. Altered cilia in the tracheal ciliary border of rabbits 30 minutes after inhalation of 2 puffs of propellants (Placebo-inhaler) and salbutamol (Ventolin)

n = 3, mean ± SD
values marked * differ significantly (α = 0.01) from controls
values marked # differ significantly (α = 0,01) from corresponding values in the group of rabbits after inhalation of Ventolin

in the epithelium. Goblet cells mostly filled with mucus were scattered among the ciliated ones. Only 3 ± 1% of them discharged their secretion by means of gradual evacuation of the individual apical mucous granules (Fig. 1). Both the conventional and lectin histochemistry revealed the dominance of goblet cells containing acid glycoconjugates. The proportion of goblet cells containing neutral and acid sulphated glycoconjugates, detected by conventional histochemistry, and the total percentage of goblet cells containing acid sialylated glycoconjugates visualised by the reactions with the lectins were given in Tab.I. In the regular ciliary border, 9.7 ± 0.3 cilia per 1 μm^2 were found. 98.8 ± 0.1% of cilia were intact. The proportions of pathological, degenerating and malformed cilia were given in Fig. 2.

After inhalation of the aerosol of propellants contained in the metered-dose inhaler, a slightly altered pseudostratified columnar ciliated epithelium was encountered in the tracheae. The intercellular spaces were narrow and the apical junctional complexes remained intact (Fig. 3).

Only a few tiny cytoplasmic protrusions exceptionally containing isolated axonemes of degenerating kinocilia were found on the apical portions of the ciliated cells (Fig. 4). A slight increase in number of small vacuoles and lysosomes was observed in the deeper portions of their cytoplasm (Figs. 5, 6). In some cells, larger intracytoplasmic ciliated vacuoles were revealed. Only isolated differentiating ciliated or secretory cells were encountered in the epithelium.

The goblet cells were mostly scattered among the ciliated ones, only 8 ± 1% of them formed tiny groups. 16 ± 1% of the goblet cells were stimulated to discharge mucus (Figs 7, 8). The proportions of mucus-discharging and exhausted secretory elements were given in Fig. 1. Mucus was evacuated from apical mucous granules. Only exceptionally, whole packets of mucous granules were detached or signs of compound exocytosis were noticed in the stimulated goblet cells. In the goblet cells secretion, neutral glycoconjugates were not revealed. The percentage of goblet cells containing the acid sulphated and lectin-detected acid sialylated glycoconjugates was presented in the Tab. I.

A slightly impaired ciliary border with 8.4 ± 0.4 cilia per 1 μm^2 was observed. The altered cilia represented only 4.4 ± 1%. The proportions of the individual types of altered cilia were given in Fig. 2. In the area of the ciliary border, neither bacteria nor clumps of inspissated secretion were observed.

DISCUSSION

On the basis of our previous experiments, a classification of the degree of injury to the airway epithelium was proposed (Konrádová, 1991). Due to the administration of two puffs of Placebo-inhaler, mostly only mild damage of this epithelium was revealed. The mucus evacuation was induced in only one sixth of all goblet cells, the mechanism of their secretion was slightly accelerated.

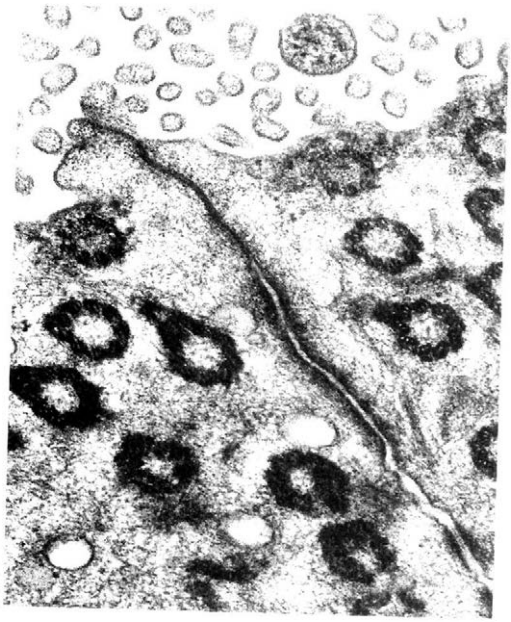
In our previous studies, we demonstrated that degeneration of more than half of the goblet cells stimulated a massive differentiation of new secretory elements resulting in their hyperplasia and changes in their distribution with the occurrence of intraepithelial mucous glands (Konrádová et al., 1990; Konrádová, 1991). As only 4% of goblet cells degenerated, the differentiation of secretory elements was not stimulated.

In accordance with other authors (Jacob and Poddar, 1982; Jeffery et al., 1982; Spicer et al., 1983; Mandal and Mandal, 1990; Castells et al., 1991, 1992), we ascertained the presence of neutral and both sulphated and sialylated glycoconjugates in the secretion of the goblet cells in healthy rabbits. The majority of acid glycoconjugates to neutral ones and the dominance of sulphated acid glycoconjugates were revealed by both histochemical and lectin-histochemistry methods. After administration of propellants, the neutral glycoconjugates disappeared from the epithelium. The composition of acid glycoconjugates produced by the goblet cells did not differ significantly from that found in healthy control rabbits. Only

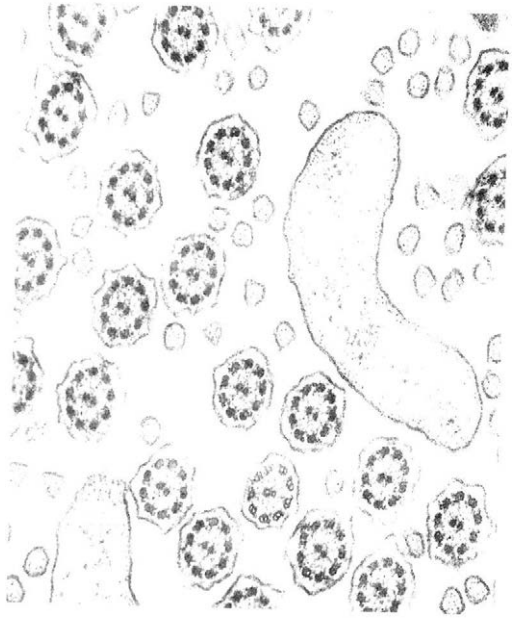
II. Evaluation of the degree of damage to the tracheal epithelium of rabbis 30 minutes after inhalation of 2 puffs of propellants (Placebo-inhaler) and salbutamol (Ventolin)

	Degree of damage to the tracheal epithelium			Control rabbits	Propellants (Placebo-inhaler)	Salbutamol (Ventolin)
	mild	moderate	severe			
Stimulated GC	3–50%	50–90%	> 90%	3%	16%	91%
Ratio $\frac{\text{discharging GC}}{\text{degenerated GC}}$	> 1	0.1–1	< 0,1	degenerated GC not found	3	0.15
Number of cilia/μm^2	7-9	3-7	< 3	9.7	8.4	5.2
Altered cilia	1.2–2.0%	2.0–10.0%	> 10.0%	1.2%	4.4%	8.6%
Signs of impairment of the self cleaning ability	±	+	++	0	0	+

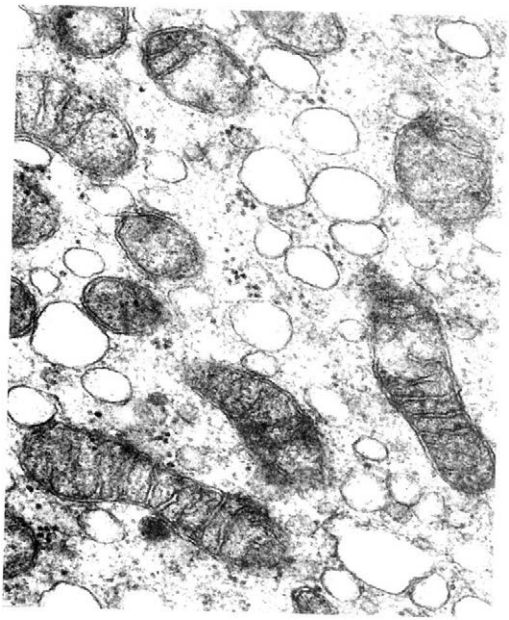
Explanation:
GC = goblet cells, 0 = no impairment of the self cleaning ability, ± = isolated bacteria in the area of the ciliary border, + = small clumps of condensed secretion and bacteria in the area of the ciliary border, ++ = voluminous clumps or layers of condensed secretion and numerous bacteria in the area of the ciliary border



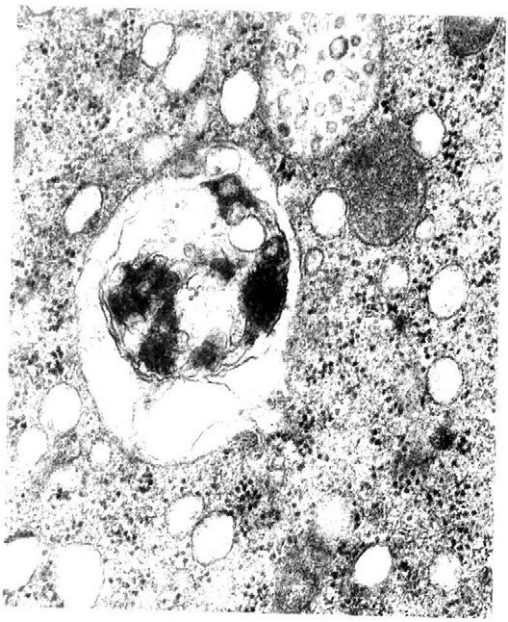
3. Apical junctional complexes between two ciliated cells. Tracheal epithelium (rabbit) – 30 min after inhalation of two puffs of Placebo-inhaler; 50 000×



4. Small apical cytoplasmic protrusions in the area of the ciliary border. Tracheal epithelium (rabbit) – 30 min after inhalation of two puffs of Placebo-inhaler; 50 000×



5. Numerous tiny vacuoles in the cytoplasm of a ciliated cell. Tracheal epithelium (rabbit) - 30 min after inhalation of two puffs of Placebo-inhaler; 50 000×



6. Larger secondary lysosome in the cytoplasm of a ciliated cell. Tracheal epithelium (rabbit) - 30 min after inhalation of two puffs of Placebo-inhaler; 50 000×

7. Apical portion of a stimulated goblet cell. Tracheal epithelium (rabbit) – 30 min after inhalation of two puffs of Placebo-inhaler; 37 500×

8. Apical portion of exhausted degenerated goblet protruding above the epithelium. Tracheal epithelium (rabbit) – 30 min after inhalation of two puffs of Placebo-inhaler; 37 000×

a slight, insignificant decrease in acid sialylated glycoconjugates, especially due to the absence of glycoconjugates sialylated at α (2–6) glycosidic linkage to galactose or N-acetylgalactosamine, was demonstrated.

As a result of Placebo-inhaler administration, the ciliated cells revealed slight signs of alteration that was reflected in the impairment of the ciliary border. In comparison with healthy control rabbits, only mild, but significant ($\alpha = 0.01$) decrease in average number of kinocilia together with an increase in number of altered kinocilia were noticed. Among the altered cilia, the slightly damaged pathological ones were the most numerous.

Morphological signs of impaired self-cleaning ability of the tracheal epithelium were not encountered (Konrádová, 1991, Stratmann et al., 1991; Konrádová et al., 1996a; Wanner et al., 1996; Geiser et al., 1997). In the area of the ciliary border, neither condensed mucus nor bacteria were observed.

To verify the theory that the damage produced in the airway epithelium by Ventolin aerosol is mostly due to the propellants contained in the metered-dose inhalers, we compared the effect of administration of Placebo-inhaler with that of Ventolin .

As we described in our previous studies (Konrádová et al., 1997, 1998), the treatment with two puffs of Ventolin aerosol, where 0.2 mg of salbutamol was contained, highly affected the secretory elements. More than 90% of goblet cells were stimulated to discharge their mucus. Mucus release was significantly accelerated and the mechanism of secretion was influenced. Signs of an apocrine type of secretion and of a rapid compound exocytosis (Specian and Neutra, 1980; Kurosumi et al., 1981; Roumagnac and Laboisse, 1987; Specian and Oliver, 1991; Konrádová et al., 1996b; Newman et al., 1996) were noticed in numerous cells. An absolute predominance of acid sulphated glycoconjugates was revealed in their secretion. After rapid release of mucus, 79% of exhausted cells degenerated and were gradually sloughed off. Rather numerous differentiating secretory elements were noticed in the epithelium, but their distribution in the epithelium was not influenced. The ciliated cells were less damaged compared with the goblet ones. Blebbing of the apical portions of their cytoplasm was associated with destruction of rather numerous kinocilia. Marked signs of pathological alteration were also noticed in the deeper portions of the ciliated cells' cytoplasm. In the area of the ciliary border, a significant ($\alpha = 0.01$) decrease in the mean number of kinocilia to 5.2/μm^2 accompanied with a significant ($\alpha = 0.01$) increase in percentage of altered cilia was recorded in comparison with healthy control rabbits. Layers of condensed secretion and bacteria representing morphological signs of impaired self-cleaning ability of the epithelium were discovered among free cilia.

After administration of propellants contained in Placebo-inhaler, significantly less pronounced ultrastructural alteration and only slight changes in the composition of glycoconjugates in the goblet cells were encountered. We are thus not able to confirm Spahr-Schopfer's and Shorten's hypothesis (Spahr-Schopfer et al., 1994; Shorten et al., 1995) that damage to the airway epithelium due to the inhalation of bronchospasmolytic drug Ventolin is mostly caused by the propellants contained in the metered-dose inhalers.

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BIOCHEMICAL MARKERS OF DIOXIN-LIKE TOXICITY AND OXIDATIVE STRESS IN HEPATIC MICROSOMES OF BREAM (*ABRAMIS BRAMA*) AND PERCH (*PERCA FLUVIATILIS*) IN THE ELBE RIVER*

BIOCHEMICKÉ MARKERY TOXICITY DIOXINOVÉHO TYPU A OXIDATIVNÍHO STRESU V CEJNU VELKÉM (*ABRAMIS BRAMA*) A OKOUNU ŘÍČNÍM (*PERCA FLUVIATILIS*) Z ŘEKY LABE

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ABSTRACT: Biochemical markers of dioxin-like toxicity and oxidative stress were investigated in hepatic microsomal fractions of bream (*Abramis brama*) and perch (*Perca fluviatilis*) collected at six sites of the Elbe river (Czech Republic). Concentrations of polychlorinated biphenyls (PCBs) and organochlorine pesticides were determined in pooled muscle samples to screen contamination rates of the sampling sites. Induction of the cytochrome P4501A-dependent 7-ethoxyresorufin *O*-deethylase (EROD) activity was used as the biomarker of dioxin-like toxicity, and the activation of microsomal glutathione *S*-transferase (mGST) and *in vitro* susceptibility to lipid peroxidation were chosen as the biochemical markers of oxidative stress in fish. The concentrations of PCBs and DDT metabolites were higher in bream than perch tissues; the EROD activity was higher in perch which were also found to be more susceptible to oxidative damage to cellular membranes measured as Fe/NADPH-enhanced *in vitro* lipid peroxidation. Exposure to dioxin-like contaminants was detected at several sampling sites at which higher concentrations of PCBs and hexachlorobenzene were determined analytically. Both the oxidative stress parameters were increased significantly at two sampling sites. At one of them, however, the results of chemical analyses indicate that the increases in lipid peroxidation and mGST activity were caused by chemicals other than the contaminants monitored within this study. The parameters of oxidative stress can be regarded as important biomarkers of toxicity suited to complete routine chemical analyses and screen the total chemical impact.

biomarkers; PCBs; organochlorines; CYP1A; dioxin toxicity; glutathione; lipid peroxidation; oxidative stress

ABSTRAKT: Biochemické markery dioxinové toxicity a oxidativního stresu byly sledovány v jaterních mikrosomech cejna velkého (*Abramis brama*) a okouna říčního (*Perca fluviatilis*) odchycených v šesti lokalitách řeky Labe. Ve svalovině ryb byly analyticky stanoveny koncentrace polychlorovaných bifenylů (PCB) a organochlorových pesticidů, aby byla zjištěna úroveň kontaminace na odběrových místech. Indukce cytochrom P4501A-dependentní 7-ethoxyresorufin-O-deetylázové aktivity (EROD) byla užita jako biomarker toxicity dioxinového typu; aktivace mikrosomální glutathion-S-transferázy a lipidní peroxidace *in vitro* byly vybrány jako biochemické markery oxidativního stresu v rybách. V tkáni cejna byly nalezeny vyšší koncentrace PCB a reziduí DDT. EROD aktivita byla vyšší u okouna, který se také ukázal citlivější k oxidativnímu poškození buněčných membrán (stanovenému jako Fe/NADPH-indukovaná lipidní peroxidace *in vitro*). Expozice kontaminanty dioxinového typu byla detekována na odběrových místech, na nichž zároveň byly stanoveny vyšší koncentrace PCB a hexachlórbenzenu. Na dvou odběrových místech byly také zvýšeny oba parametry oxidativního stresu; na jednom z nich však pravděpodobně byla modulace těchto biomarkerů způsobena kontaminanty, které nebyly analyticky sledovány. Stanovení parametrů oxidativního stresu a biomarkeru dioxinové toxicity ukázalo, že data z biochemického monitorování vhodně doplňují rutinní chemické analýzy a sledují celkový impakt chemických látek na organismus.

biomarkery; PCB; organochlorové kontaminanty; CYP1A; dioxinová toxicita; lipidní peroxidace; oxidativní stres

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INTRODUCTION

The objective of biochemical monitoring is to assess actual toxic effects of contamination by measuring responses of specific biochemical parameters (biomarkers) to the most important modes of toxic action. Along with chemical analyses of some major groups of contaminants (Peakall and Walker, 1994; van Gestel and van Brummelen, 1996), this approach to the monitoring of aquatic pollution is becoming a part of ecotoxicological risk assessment. Only some toxicity mechanisms, such as dioxin-like toxicity and oxidative stress (Stegeman and Hahn, 1994; Kelly et al., 1998), have so far been measured *in vivo* to demonstrate chemical stress.

The dioxin-like toxicity includes effects, whose activation is connected with the interaction of the toxicant with a certain cellular receptor (Ah-receptor). This interaction results in increased metabolic activation of promutagens and procarcinogens, increased cellular proliferation, neurotoxicity, endocrine dysbalance, etc. (Safe, 1990; van den Berg et al., 1998). A typical compound with such effects is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); the respective biochemical marker is the induction of cytochrome P4501A and an increase in the specific activity of this enzyme, which is often determined as 7-ethoxyresorufin *O*-deethylase (EROD).

Oxidative stress is defined as a dysbalance between the natural production of prooxidants and the levels of antioxidant defence systems. An overproduction of prooxidants arises from an exposure to noxious xenobiotics. Various modes of action can be involved in this process (Klaunig et al., 1998). Oxidative stress is thus one of the important manifestations of general stress caused by pollutants. The activation of the liver microsomal antioxidative enzyme glutathione *S*-transferase (mGST) is apparently an effective biomarker of an intensification of prooxidative processes pursuant to an exposure to xenobiotics in fish. This activation is an adaptation mechanism; mGST is rather activated by reactive oxygen species than synthesised *de novo* (Moorhouse and Cassida, 1992). Our previous laboratory and field studies (Petřivalský et al., 1997; Machala et al., 1998) have shown that the increase in the activity of hepatic mGST in some fish species correlates well with an intensification of prooxidative processes after an exposure to aquatic contaminants, such as organochlorine pesticides.

Exposures to xenobiotics are followed by changes in the levels of certain enzymes involved in prooxidative processes. Such changes can result in increased susceptibility of membrane lipids to oxidative damage as indicated by the level of Fe(II)/NADPH-induced lipid peroxidation *in vitro* in the hepatic microsomal fraction (Machala et al., submitted for publication).

The objective of this study was to determine the impact of dioxin-like contaminants and to compare levels and modulations of oxidative stress biomarkers in hepatic tissue of bream and perch captured at different sites of the Czech part of the Elbe river.

MATERIAL AND METODS

Chemicals

7-ethoxyresorufin was obtained from Molecular Probes (Eugene, MI, USA), resorufin, NADPH, bicinchoninic acid, reduced glutathione, 1-chloro-2,4-dinitrobenzene, thiobarbituric acid, and 1,1,3,3-tetraethoxypropane were purchased from Sigma-Aldrich (Prague, Czech Republic). Organochlorine and PCB congeners and isomers were obtained from Dr. Ehrenstorfer (Darmstadt, Germany). All other chemicals were of the highest commercially available purity.

Sampling, chemical and biochemical analyses

Bream (*Abramis brama*) and perch (*Perca fluviatilis*) were captured at six sites of the Elbe river in August 1996. After cervical dislocation, hepatic and muscular tissues were sampled for the measurement of biochemical toxicity markers and chemical analyses, respectively. The samples were frozen on dry ice, transported to lab in less than 24 hr, and stored at –80 °C. The age of fish was estimated by scale analysis.

Dorsal muscle samples (approximately 5 g/fish) collected at each site were pooled and homogenised before the analysis. The pooled samples were analysed for the contents of the major classes of persistent chlorinated contaminants using conventional analytical methods including Soxhlet extraction with petroleum ether-acetone, clean-up on a gel permeation column, and fractionation in a HYPERCARB column (Shandon, Runcorn, U.K.). Indicator and mono-*ortho*-chlorinated PCBs (IUPAC Nos. 28, 52, 101, 105, 118, 138, 153, 156, and 180), DDT and their residues, hexachlorobenzene and hexachlorocyclohexane were determined by HRGC/ECD (Machala et al., 2000).

Individual hepatic tissue samples were homogenised and microsomal fraction was separated by differential centrifugation as described previously (Petřivalský et al., 1997). Thereafter, it was stored at –80 °C until the biochemical analysis. The EROD activity was determined fluorimetrically (Prough et al., 1978); the final concentration of the substrate 7-ethoxyresorufin was 2 μM. The conjugation activity of microsomal GST was determined spectrofluorometrically with 1-chloro-2,4-dinitrobenzene as the substrate (Moorhouse and Cassida, 1992). All the enzyme activities were determined at 30 °C.

Lipid peroxidation was induced *in vitro* by Fe(II)/NADPH (final concentrations of $FeSO_4$ and NADPH were 0.33 mM and 0.36 mM, respectively) in hepatic microsomes for 30 min and determined spectrophotometrically

with thiobarbituric acid (Uchiyama and Mihara, 1978). Protein concentration was measured using the bicinchoninic acid assay (Smith et al., 1985).

RESULTS AND DISCUSSION

The concentrations of polychlorinated biphenyls (PCBs) and the organochlorine pesticides hexachlorobenzene (HCB), hexachlorohexane (HCH) and DDT and their metabolites were determined in pooled bream and perch muscle samples collected at each of the six sampling sites in the Czech part of the Elbe river. Sampling data and concentrations of the contaminants are summarised in Tab. I.

The results of chemical analyses indicate that fish were contaminated by PCBs already at site 1 situated upstream from the Pardubice industrial area. Significantly higher PCB contamination rates were found at sites 5 and 6, but even here the concentrations in muscle tissue were below maximum value tolerated in the Czech Republic. The concentrations of DDT, incl. its metabolites, and hexachlorobenzene at sites 5 and 6 were also rather high. On the other hand, lindane and other HCH isomers were present at very low concentrations only (maximum of 0.002 μg/kg fresh weight at site 4, data not shown). The bioaccummulation of chlorinated aquatic contaminants was apparently higher in bream than in perch muscle tissues.

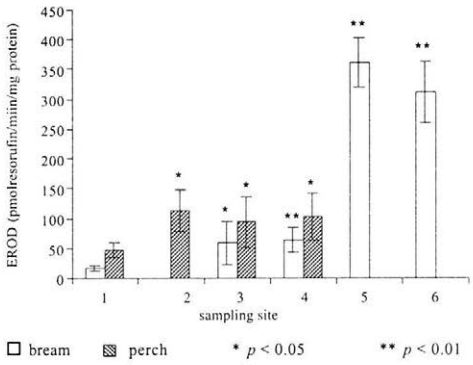
Candidate biochemical *in vivo* markers should indicate exposure to or specific noxious effects of aquatic contaminants. Only such biochemical parameters are considered suitable whose modulation due to chemical contamination is sensitive enough. In view of a variety of mechanisms of adverse effects of xenobiotics, one biomarker is insufficient and it will be necessary in the near future to make up a battery of sensitive biomarkers responding to major modes of action of contaminants including dioxin-like toxicity and oxidative stress. In our study, biochemical markers in individual hepatic microsomal fractions of bream and perch from the same sampling sites were induced in a very similar way (Figs. 1–3). Sampling site 1 was taken as a reference site with a low contamination rate. The EROD activity, as the specific biomarker of exposure to planar organic contaminants with dioxin-type toxic potencies, was significantly increased at sites 5 and 6 which were heavily contaminated by persistent chlorinated chemicals. EROD induction was found also at sites 2–4 where only moderate contamination by these compounds was demonstrated by chemical analyses (Fig. 1). This finding agreed well with the results of previous studies where this biomarker was shown to be sensitive to coplanar PCB congeners in a number of fish species (Stegeman and Hahn, 1994). The induction of hepatic microsomal EROD activity in bream was studied as a biomarker of dioxin-like toxicity along with the determination of PCB and Hg concentration in liver tissue of fish collected in the lower course of the Elbe river in Germany (Jedamski-Grymlas et al., 1995). Like in our study, the induction of the EROD activity was associated with higher concentrations of PCBs. However, the EROD induction threshold for PCBs and other dioxin-like contaminants and the induction variability of this biomarker are still to be estimated in fish species coming into consideration for biochemical monitoring. Remarkably, in our study, the EROD activities were significantly higher in perch than in bream (Fig. 1).

Besides the contribution from PCBs, when considering which organic contaminants might induce the EROD activity in bream and perch, HCB has also a potency to dioxin-like activity in the concentration of 20 ng HCB per kg wet weight. Compared with the reference toxicant TCDD, its toxic equivalency factor is 0.0001 (van Birgelen, 1998), which is a value similar to that of mono-*ortho*-chlorinated PCBs (van den Berg et al., 1998). Moreover the presence of other dioxin-like toxicants, halogenated dibenzo-*p*-dioxins or dibenzofurans, which were not determined analytically, must also be considered. In any case, EROD was definitely found to be a suitable parameter complementary to chemical analysis and its induction indicated a risk of contamination by planar dioxin-like contaminants, especially at sites 5 and 6.

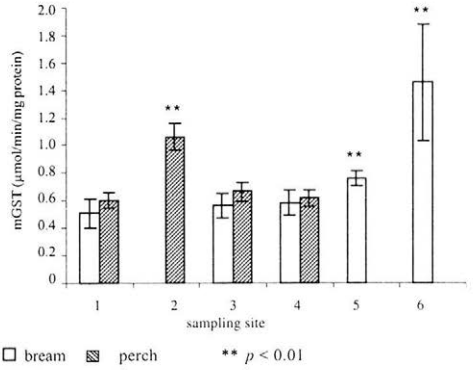
The two oxidative stress parameters under study, namely the microsomal GST activity and the Fe(II)/NADPH-enhanced *in vitro* lipid peroxidation, were significantly increased at sampling sites 2 and 6 (Figs. 2, 3).

I. Sampling sites, number of samples and concentration of contaminants in pooled fish muscle (mg/kg fresh weight). Distances of the sampling sites are expressed in km (measured upstream)

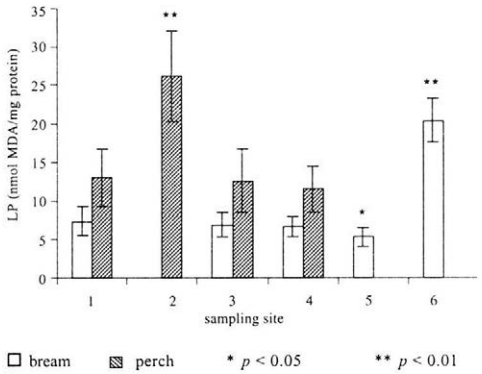
	Sampling site (km)	Species (*n*)	Age	Σ PCB	Σ DDT + DDE	HCB
1	Opatovice (262.6)	bream (5)	5–6	0.111	0.030	0.001
		perch (8)	2–5	0.038	0.010	0.001
2	Valy (224.6)	perch (7)	3–4	0.096	0.008	0.002
3	Lysá n. L. (150.7)	bream (4)	3–7	0.174	0.018	0.003
		perch (3)	4–7	0.069	0.008	0.002
4	Obříství (116.2)	bream (5)	4–8	0.187	0.026	0.002
		perch (10)	5–8	0.050	0.007	0.001
5	Děčín (12.0)	bream (4)	6–9	0.307	0.093	0.023
6	Hřensko (0)	bream (4)	5–7	0.251	0.087	0.028



1. Hepatic microsomal CYP1A-dependent 7-ethoxyresorufin *O*-deethylase (EROD) activities in bream and perch collected at six sampling sites in the Elbe river (mean ± SD); for the description of sampling sites and sample sizes see Tab. I



2. Microsomal glutathione *S*-transferase activities toward 1-chloro-2,4-dinitrobenzene (mGST) in liver of bream and perch in the Elbe river (mean ± SD); for sampling sites see Tab. I



3. Fe(II)/NADPH-dependent *in vitro* lipid peroxidation in hepatic microsomal fraction of bream and and perch determined as thiobarbituric acid-reactive species (mean ± SD); for sampling sites see Tab. I)

A comparison of analytical data with increased values of the biomarkers at the two sampling sites let us assume that oxidative stress found at site 2 was probably caused by other than the monitored contaminants. Antioxidant enzymes have been proposed as biochemical markers of noxious prooxidative processes for environmental risk assessment (Winston and DiGiulio, 1991; Kelly et al., 1998). The microsomal GST is a membrane-bound antioxidant enzyme. An increased mGST activity was described in marine fish from contaminated littoral sites (Rodríguez-Ariza et al., 1993), carp collected from contaminated lakes (Machala et al., 1997), and rainbow trout exposed to model oxidative stressors (Petřivalský et al., 1997; Machala et al., 1998).

The activation of mGST directly correlates with the increase of prooxidative processes (Petřivalský et al., 1997; Machala et al., 1998). On the other hand, *in vitro* lipid peroxidation in the hepatic microsomal membrane is not an unambiguous parameter as shown in a recent field study in chub (Machala et al., submitted for publication). Increased or decreased lipid peroxidation *in vitro* can be found in hepatic microsomal samples of fish collected at more contaminated sites. Compared with reference site 1 (Fig. 3), the *in vitro* lipid peroxidation in the present study was lower in the samples collected at site 5 and higher in those collected at site 6.

This difference in the modulation between the two oxidative stress parameters can be explained as a result of increased capacity of antioxidant systems accompanied by a less severe damage to membrane phospholipids (Rodríguez-Ariza et al., 1993). In this study, the lipid peroxidation *in vitro* in the hepatic microsomal fraction was higher in perch than in bream at all the sampling sites; this difference may have been be associated with a higher susceptibility of cell membrane to oxidative damage in the former species.

In conclusion, the hepatic microsomal EROD activity was shown to be a suitable biomarker responding to the dioxin-like toxicity in both bream and perch. The activation of mGST was found to be a sensitive biochemical marker of oxidative stress in these fish species. Practical application of Fe(II)/NADPH-enhanced *in vitro* lipid peroxidation as another candidate oxidative stress indicator will require a more detailed explanation of its modulation mechanisms.

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Změna publikačního jazyka ve vědeckých časopisech ČAZV

Předsednictvo České akademie zemědělských věd přijalo na zasedání dne 6. 4. 2000 usnesení, kde mj. doporučuje změnu publikačního jazyka ve vědeckých časopisech vydávaných pod gescí ČAZV. Předsednictvo navrhuje Vydavatelské radě ČAZV zavést angličtinu jako jediný jazyk ve všech vědeckých časopisech od 1. 1. 2001.

Od 1. 7. 2000 redakce časopisu Veterinární medicína přijímá příspěvky psané pouze v angličtině. Příspěvky přijaté do tohoto termínu budou ještě uveřejněny v češtině a slovenštině.

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A change of publication language in Scientific Journals of the Czech Academy of Agricultural Sciences

At its session on the 6th April 2000, the Presidium of the Czech Academy of Agricultural Sciences adopted a resolution recommending, among other things, to change the publication language in scientific journals published under the Academy patronage. The Presidium proposes to the Publishing Board of the Academy to introduce English as the only language in all scientific journals from the 1st January 2001.

The papers written exclusively in English are accepted by the editor's office of the journal Veterinary Medicine - Czech from the 1st July 2000. The papers that were accepted before this date will be published in Czech and Slovak.

EFFECTS OF HEAT-PROCESSING OF MILK ON VALIDATION CHARACTERISTICS OF THE CHARM AIM-96 AND DELVOTEST SP SCREENING TESTS

VLIV TEPELNÉHO OŠETŘENÍ MLÉKA NA CHARAKTERISTIKY VALIDACE SCREENINGOVÝCH TESTŮ CHARM AIM-96 A DELVOTEST SP

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ABSTRACT: Two commercial screening tests (Charm AIM-96 and Delvotest SP) for the detection of antimicrobial residues in milk were evaluated using samples of heat-processed milk. Heating to 85 °C for 5 min had no effect on detection limits for selected antimicrobial agents diluted in milk including penicillin, streptomycin, neomycin, chlortetracycline, cloxacillin, chloramphenicol and sulphadiazine. Heat degradation of the interfering natural inhibitors (lysozyme, IgG) resulted in considerable changes of almost all characteristics of validation of raw and heat-processed milk in both the tests. The value of true specificity increased from 96.55% for Charm AIM-96 and 90.62% for Delvotest SP to 100.0%. The most marked change occurred in the cutoff specificity which is decisive for correct scoring of milk with the concentrations of antimicrobial residues <MRL (maximum residue limit) as negative. Heat processing reduced the number of false positive samples detected by Delvotest SP by up to 18.21% ($P < 0.01$). The characteristics of "cutoff sensitivity" were unaffected. Both raw and heat-processed milk samples containing residues of antimicrobial agents at levels corresponding of MRL were scored correctly by both the tests. Heat inactivation of milk samples before screening tests, used usually for bulk milk samples, is necessary considering the intended checks of individual milk samples for residues of antimicrobial agents.

milk; residues; antimicrobial drug; lysozyme; degradation

ABSTRAKT: Dva komerčně dostupné screeningové testy Charm AIM-96 a Delvotest SP pro stanovení reziduí antimikrobiálních látek v mléce byly evaluovány s přihlédnutím k vlivu tepelného ošetření mléka před testací. Detekční limity testů pro vybrané antimikrobiální látky, a to penicilin, streptomycin, neomycin, chlortetracyklin, cloxacilin, chloramfenikol a sulfadiazin, byly shodné při použití mléka tepelně ošetřeného (85 °C, 5 min) před přípravou vzorků (HB) a po tepelném ošetření vzorků s antimikrobiálními látkami (HA). Tepelná denaturace přirozených inhibitorů v mléce (lysozymu, IgG) způsobila v důsledku diferenci hodnot charakteristik validace při použití syrového mléka oproti tepelně ošetřenému mléku (HA a HB) u obou testů. Hodnota charakteristiky "true specifity" testů vzrostla pro test Charm AIM-96 z 96,55 % a pro test Delvotest SP z 90,62 % ($P < 0,01$) na 100 % u obou testů. Identifikace vzorků bez reziduí a s rezidui antimikrobiálních látek pod povolenými maximálními limity reziduí (MRL) jako negativní ("cutoff specifity"), byla ovlivněna významně u testu Delvotest SP ($P < 0,05$ a $P < 0,01$) i u testu Charm AIM-96 ($P < 0,05$). Schopnost testů detekovat vzorky s rezidui antimikrobiálních látek nejméně na úrovních MRL ("cutoff sensitivity") zůstala nezměněna. Schopnost testu Delvotest SP odlišit negativní vzorky od vzorků pozitivních se významně zvýšila ($P < 0,05$). Tepelná inaktivace vzorků před provedením screeningových testů, obvykle používaných pro směsné vzorky, je obzvláště nutná pro detekci reziduí antimikrobiálních látek v individuálních vzorcích mléka.

mléko; rezidua; antimikrobiální látka; lysozym; degradace

INTRODUCTION

Treatment of food animals with antimicrobial agents has given rise to problems resulting from the presence of drug residues in edible tissues. Recent surveys carried out in the Czech Republic repeatedly demonstrated the presence in raw milk of β-lactam, aminoglycoside, and tetracycline antibiotics in up to 0.5% of the samples tested in the central laboratories (SCL, 1999). Research has concentrated not only on adverse effects of such drugs on human health (Dewdney and Edwards, 1984; Kidd, 1995) and attempts to remove from foods (Charm, 1980),

but particularly on the development of effective residue detection methods. In most laboratories (Havlová et al., 1993; Suhren, 1995), routine tests are carried out using microtitre plate methods based on growth inhibition of the test strain C-953 (ATCC 10149-IDF, 1991) of *Bacillus stearothermophilus* var. *calidolactis*, such as Intest – Czech Republic, BR tests – Germany, Delvotest P – The Netherlands, and Charm AIM-96 test – USA. The presence of inhibitory agents is revealed by absence of a colour reaction of an indicator on metabolites of the growing test strain.

Various integrated systems for the monitoring of residues of antimicrobial agents have been suggested in recent years. They include both rapid screening methods without specifying the agent, and tests confirming the identity and determining the concentration of the chemical agent. "US Safe/Tolerance Levels" and "Maximum Residue Levels" (MRL) for drug residues have been laid down in the USA and the European Communities, respectively. Screening methods detecting a broad spectrum of antimicrobial agents at such levels are preferred as the first step of any integrated system of residue monitoring (Heeschen and Suhren, 1995).

The milk processing stage at which the test is to be applied must be considered in the selection of the method. The results of validation and/or operation of the methods can be significantly affected by several factors. Thus, for instance, the results of screening tests for β-lactam antibiotics can be influenced by factors used as parameters for milk quality assessment (total microorganism, coliform bacteria, and milk somatic cell counts), and by characteristics of milk production, such as parity, day of lactation and milking performance (Andrew et al., 1997). Such effects were demonstrated, among other authors, by Van Eenennaam et al. (1993) who used the screening methods CITE® probe (β-lactam), Delvotest-P®, Charm Farm®, Lac Tek®, and the diffusion method with *Bacillus stearothermophilus* var. *calidolactis* to test individual milk samples collected form cows suffering from clinical mastitis. Before the sampling, the cows were treated with drugs containing amoxicillin, cefapirin, or oxytocine. False positive tests were obtained in four tests in samples containing increased milk somatic cell counts.

Another factor influencing the results of screening tests is the presence in milk of natural inhibitors of microbial growth. The occurrence of false positive results of the Delvotest P screening method correlates well with increased concentrations of lactoferrin and lysozyme in milk (Carlsson and Björck, 1989). Therefore, heat inactivation of natural inhibitors has been recommended (IDF, 1991). Such recommendation has not been included in instructions for use provided by the manufacturers of Charm AIM-96 and Delvotest SP, however.

The aim of this study was to verify the detection limits of Charm AIM-96 and Delvotest SP and to assess elementary characteristics of the two tests after heat inactivation of the natural inhibitors in milk samples.

MATERIAL AND METHODS

Milk

Milk intended for the dilution of antimicrobial agents was collected aseptically from a healthy, untreated cow after lactation day 182 of the eighth lactation period. Analyses done during the experimental period (10 days) showed the following values: fat 2.33%; proteins 3.37%; lactose 4.32%; SCC 50 000 to 191 000; pH 6.70 to 6.86; IgG by radial diffusion technique (Mancini et al., 1965) 1.0 to 1.9 mg/ml; lysozyme by radial diffusion technique with *Micrococcus lysodeicticus* (Richter, 1986) 3.8 to > 4.0 μg/ml.

Samples

Three series of samples were prepared for each of the seven antimicrobial agents and for each day of the experiment as follows: antimicrobial agents diluted in raw milk (RM); antimicrobial agents diluted in raw milk which was subsequently heat-treated (HA); antimicrobial agents diluted in heat-treated milk (HB). The treatment consisted in heating to 85 °C for 5 min and immediate cooling to 20 °C.

Antimicrobial drugs

The antimicrobial agents, their dilution series and approved MRL are summarised in Tab. I. The selection of the agents was done considering the frequency of their use in drugs intended for therapy of mastitis in the Czech Republic. Chloramphenicol is present in one of them, but its use for the treatment of food animals has been limited by the World Health Organisation since 1969 (WHO, 1969). Checks for illegal use are necessary.

Screening tests

Two screening tests for the detection of residues of antimicrobial agents in milk were validated – Charm AIM-96 test (Charm Sciences, Inc., USA) and Delvotest SP (Gist-brocades Food Ingredients Inc., The Netherlands). The manufacturers' instructions for the preparation of microplates and execution of the tests were strictly adhered to.

For the Charm AIM-96 test, the freeze-dried nutrient medium with the pH indicator, and a tablet containing the bacterial culture were reconstituted in the attached solution. This mixture was then pipetted into wells of microtitre plates containing the milk samples to be tested. The plates were then incubated in an appliance supplied by the manufacturer of the test kit.

For the Delvotest SP, tablets of the bacterial culture were put into individual microtitre plate wells containing

nutrient agar with pH indicator. The tablets dissolved after the addition of milk samples into the wells. The plates were incubated in a water bath at 64 °C for 2.5 to 3 h. The length of the incubation period depended on the development of yellow colour in the negative control well.

Raw and heat-treated milk free of microbial agents were used as negative controls which were run in each microtitre plate along with the samples containing the antimicrobial agents. The numbers of the negative controls for Charm AIM-96 were 29 raw milk and 34 heat-treated milk samples. The corresponding numbers for Delvotest SP were 32 and 42, respectively. The original positive control supplied by Charm, containing 4 μg penicillin and 50 μg sulphamethazine per litre, as well as a negative control (heat-treated milk without antibiotics) were run in each plate.

The Charm AIM-96 test and Delvotest SP were repeated 7 and 8 times, respectively.

Evaluation

The evaluation of the reactions was done according to the manufacturers' recommendations with the exception that all samples showing at least a weak reaction were scored as positive. Moreover, the detection limits and detectability of MRL (the potential of the method to detect antimicrobial agents at least at MRL) were assessed.

The following characteristics were calculated for each test and each variant of milk treatment:

$$\text{"true" specificity [\%]} = [TN/(TN+FP)] \cdot 100 \quad (1)$$

where:
TN = "true" negative samples (free of antimicrobial agents),
FP = "false" positive samples (free of antimicrobial agents, but yielding positive reactions);

$$\text{"cutoff" specificity [\%]} = [CN/(CN+CFP)] \cdot 100 \quad (2)$$

where:
CN = "cutoff" negative samples (free of antimicrobial agents or containing antimicrobial agents at concentrations lower than the "cutoff" value, where "cutoff" = MRL for the given agent);
CFP = "cutoff" false positive samples (free of antimicrobial agents or containing antimicrobial agents at concentrations lower than the "cutoff" value, but showing positive reactions).

The parameters define the potential of the method to identify correctly samples free of antimicrobial agents and samples containing residues of antimicrobial agents at concentrations lower than MRL as negative.

$$\text{"cutoff" sensitivity [\%]} = [CP/(CP+CFN)] \cdot 100 \quad (3)$$

where:
CP = "cutoff" positive samples (containing antimicrobial agents at MRL),
CFN = "cutoff" false negative samples (containing antimicrobial agents at the level of MRL, but yielding negative reactions).

The parameter defines the potential of the method to identify samples containing residues of antimicrobial agents at least at MRL as positive.

$$\text{"predictive value" of positive tests [\%]} = [CP/(CP+CFP)] \cdot 100 \quad (4)$$

$$\text{"predictive value" of negative tests [\%]} = [CN/(CN+CFN)] \cdot 100 \quad (5)$$

$$\text{"cutoff" efficacy [\%]} = [(CP+CN)/(CP+CFP+CN+CFN)] \cdot 100 \quad (6)$$

The parameters define the potential of the method to distinguish samples free of residues of antimicrobial agents and samples containing residues of antimicrobial agents below MRL from samples containing residues at or above MRL.

An ideal test should reach 100% in all the above parameters (Deshpande, 1996).

Differences in the characteristics of the individual tests between the raw milk and heat-treated milk samples were analysed by the χ^2 test (Armitage and Barry, 1987).

RESULTS

A decrease of lysozyme and IgG levels to ≤ 0.2 mg/ml and ≤ 1.0 mg/ml, respectively, was observed after heating the milk to 85 °C for 5 min and immediate cooling to 20 °C.

The ranges of detection limits for the individual antimicrobial agents and the three variants of milk as the diluting medium are summarised in Tab. II. The detection limits corresponding to the lowest value in the dilution series include also limits below this value which were not

I. Selection of antimicrobial agents, dilutions used in the tests and MRL values for milk

Antimicrobial agent*	Dilution (μg/l)			MRL (μg/l)
Benzylpenicillin potassium salt	6	4	2	4
Streptomycin sulphate	500	200	100	200
Neomycin sulphate	500	200	100	500
Chlortetracycline hydrochloride	500	100	50	100
Cloxacillin sodium salt	50	30	10	30
Chloramphenicol	6000	1000	500	0
Sulphadiazine	500	100	50	100

*antimicrobial agents (Sigma-Aldrich Ltd)

II. Detection limits of Charm AIM-96 and Delvotest SP for selected antimicrobial agents

Antimicrobial agent	Detection limits (μg/l)					
	Charm AIM-96			Delvotest SP		
	RM	HA	HB	RM	HA	HB
Penicillin	2–4	2–4	2–4	2	2	2
Streptomycin	200–500	≥ 500	≥ 500	200–≥ 500	≥ 500	> 500
Neomycin	100	100	100	200–500	≥ 500	≥ 500
Chlortetracycline	50–500	100–500	100–500	50–500	100–500	100–500
Cloxacillin	10–30	30	30	10–50	30	10–30
Chloramphenicol	1 000–6 000	≥ 6 000	≥ 6 000	500–6 000	500–6 000	1 000–6 000
Sulphadiazine	50	50	50	50–100	50–100	50–100

RM – raw milk; HA – milk heat-processed after the addition of the antimicrobial agent; HB – milk heat-processed before the addition of the antimicrobial agent

examined. It can be seen that the detection limits for penicillin were equal to, or lower (Delvotest SP) than MRL (Tab. I). Residues of chloramphenicol were detected by any of the tests only at concentrations ≥ 500 μg/l. The sensitivity of the two tests for cloxacillin, sulphadiazine, chlortetracycline, and insensitivity for streptomycin (≥ 500 μg/l) were identical or almost identical when HA or HB samples were tested. The sensitivity for neomycin was lower in Delvotest SP than in Charm AIM-96 test (200 to ≥ 500 vs. 100 μg/l).

Heat treatment of milk did not affect the activity of the antimicrobial agents, as demonstrated by equal detection limits in milk samples treated before or after the addition of the agents (HA vs. HB).

The number of samples containing residues of antimicrobial agents at MRL and scored as positive are given in Tab. III. Both tests reliably identified milk samples containing penicillin, cloxacillin, and sulphadiazine at MRL in both heat-treated milk (100%) and raw milk (75 and 100% for Delvotest SP and Charm AIM-96, respectively), and were insensitive for chloramphenicol (0%). Residues of streptomycin at MRL were detected in only one sample of raw milk. Residues of chlortetracycline were detected by Charm AIM-96 more effectively (43% for all milk treatments) than by Delvotest SP (25, 12, and 25% for RM, HA, and HB, respectively). A considerable difference between the tests was found in the demonstration of neomycin residues. While Charm AIM-96 identified all samples at the level of 100 μg/l (below MRL) irrespective of the milk treatment, the efficacy of Delvotest SP was only 75, 50 and 50% for RM, HA, and HB, respectively. The detection limit was≥ 500 μg neomycin/l, however.

Tab. IV summarises basic validation characteristics of the screening tests for the three milk treatments, as well as results of statistical analysis of between-treatment differences. All differences were tested, but only those significant at $P < 0.05$ or $P < 0.01$ are presented.

It is evident that Charm AIM-96 identified actually negative RM samples more effectively than Delvotest SP (96% vs. 90%). Both tests identified correctly all heat-treated samples that were free of antibiotics (100% both). Heat treatment contributed to correct identification of actually negative samples, particularly when Delvotest SP ($P < 0.01$) was used, and also to correct identification of "cutoff" negative samples by Charm AIM-96 (76.32 and 77.11%, $P < 0.05$) and particularly by Delvotest SP (83.33 and 85.71%, $P < 0.01$) Charm AIM-96 proved to be more reliable for the detection of antimicrobial agents at MRL than Delvotest SP (63.26 to 65.30% vs. 51.79 to 57.14%) in RM, HA, and HB milk samples. Negative samples were identified more reliably than positive samples

III. Detectability of MRLs by Charm AIM-96 (in 7 repeated assays) and by Delvotest SP (in 8 repeated assays)

Antimicrobial agent	Percentage of recorded MRL values					
	Charm AIM-96			Delvotest SP		
	RM*	HA*	HB*	RM	HA	HB
Penicillin	100	100	100	100	100	100
Streptomycin	14	0	0	12	0	0
Neomycin	100	100	100	75	50	50
Chlortetracycline	43	43	43	25	12	25
Cloxacillin	100	100	100	75	100	100
Chloramphenicol	0	0	0	0	0	0
Sulphadiazine	100	100	100	100	100	100

*For abbreviations see Tab. II

IV. Effects of heat-processing on characteristics of validation of the screening tests Charm AIM-96 and Delvotest SP and significance (P) of the differences between raw milk (RM) and heat-processed milks (HA, HB)

Test characteristics		Tests					
		Charm AIM-96			Delvotest SP		
		RM*	HA*	HB*	RM	HA	HB
True specifity	(%)	96.55	100	100	90.62	100	100
significance							$P < 0.01$
Cutoff specifity	(%)	61.97	77.11	76.32	67.50	85.71	83.33
significance			$P < 0.05$			$P < 0.01$	$P < 0.05$
Cutoff sensitivity	(%)	65.30	63.29	63.26	57.14	51.79	53.57
Predictive value of positive test	(%)	54.24	62.00	63.27	55.17	67.44	66.67
Predictive value of negative test	(%)	72.13	78.05	76.32	69.23	75.68	74.26
Cutoff efficacy	(%)	63.33	71.97	71.20	63.23	73.38	71.19
significance						$P < 0.05$	$P < 0.05$

* For abbreviations see Tab. II

irrespective of the test used (69.23 to 78.05% vs. 54.24 to 67.44%). The efficacy of both tests was almost identical for all the variants of milk treatment, but heat treatment apparently contributed to correct identification particularly when Delvotest SP was used ($P < 0.05$ and $P < 0.05$).

DISCUSSION

The effects of heat treatment of milk samples on validation characteristics of the screening tests Charm AIM-96 and Delvotest SP for the determination of antimicrobial drug residues in milk were investigated. Conventional characteristics of screening tests, including detection limit, "true" specificity, and "predictive value" of positive and negative results, were completed with "cutoff" specificity, "cutoff" sensitivity and "cutoff" efficacy of the tests.

Investigations have demonstrated a high sensitivity of both tests for β-lactam antibiotics. The detection limit for penicillin 2 to 4 μg/l corresponded to data supplied by the manufacturers and results of interlaboratory trials of routine tests (Suhren et al., 1995). Although most of the results were false positive considering MRL laid down with regard to the protection of human health, the high sensitivity of the tests is beneficial for the dairy industry using very sensitive technological cultures. A high efficacy in the detection of β-lactam antibiotic residues at the tolerance and safety levels was demonstrated by comparison with results of liquid chromatography of milk samples collected from cows with clinical mastitis which were collected before, during, and after the treatment with amoxicillin or penicillin. Anderson et al. (1998) demonstrated agreement of results of the screening test and liquid chromatography in up to 94% of the samples.

Charm AIM-96 detected residues of neomycin already at the concentration of 100 μg/l, i.e. five times lower than the temporarily accepted MRL (500 μg/l). However, microbial cultures used in the dairy industry are more sensitive to aminoglycoside antibiotics being inhibited already at concentrations of 40 to 200 μg/l (Kraack and Tolle, 1967). Neomycin is an active component of several drugs (Vademecum, 1999) intended for the treatment of bovine mastitis. A more frequent occurrence of neomycin residues in milk can therefore be expected. Considering the data given above, Charm AIM-96 should be preferred to Delvotest SP in milk testing, although the result may be false positive in terms of MRL.

The two tests are almost equivalent as far as sensitivity for chlortetracycline and sulphadiazine is concerned. The detection limits for the latter were lower than MRL; this finding applied particularly to Charm AIM-96. Our results agree with data published for similar antimicrobial agents (Suhren et al., 1995; Charm and Zomer, 1995). In our investigations, Charm AIM-96 proved to be less sensitive for the detection of sulphonamide residues than claimed by the manufacturer, but residues below MRL were still detectable.

Chloramphenicol and streptomycin at MRL were undetectable by any of the tests. The same insensitivity was described also in other conventional screening tests (Charm and Zomer, 1995), which means that samples containing residues of these antibiotics at concentrations exceeding MRL are not identified as positive by the tests. A new systemic approach will be necessary to cope with this problem.

Heat treatment had no apparent effects on detection limits of the two tests. Lower values obtained in tests of raw milk can be attributed to the combined action of the antimicrobial agents and natural inhibitors present in milk.

The test characteristics were validated using a system common in the interpretation of results of enzymoimmunoanalytical tests in clinical diagnostics (Deshpande, 1996). The "cutoff value" ("decision value", "reference value") was identical with MRL.

The requirement to detect antimicrobial agents at MRL was met better by the Charm AIM-96 test that identified

reliably all samples containing penicillin, neomycin, cloxacillin, or sulphadiazine. The results obtained by any of the tests in RM samples containing streptomycin can be scored as false positive.

It is evident from the results that heat treatment of samples resulted in changes of all the validation characteristics excepting "cutoff" sensitivity. It is probable that inactivation of lysozyme was responsible for these changes, particularly in Delvotest SP.

The decrease in lysozyme concentration observed in heat-treated samples agrees with the results of Weaver and Kroger (1978) who demonstrated a degradation of this enzyme at 80 °C and higher temperatures. Carlsson and Björck (1987) observed an inhibition of pH shift due to the growth of *Bacillus stearothermophilus* var. *calidolactis* already at concentrations ≥ 0.1 μg lysozyme per 1 ml broth. However, the concentration of 1000 μg/ml was necessary to influence the results of Delvotest P Multi. The authors attribute this difference to different physical and chemical conditions in the agar medium, but point out that synergetic inhibitory activity of lysozyme and lactoferrin at their physiological concentrations in milk can affect the results of Delvotest P.

A decrease of milk lysozyme level due to heat treatment, observed in our experiment, was also described in the experimental study published by Weaver and Kroger (1978). Heat treatment of milk before testing for residues does not cause any significant change in the concentration of antimicrobial agents.

Heat inactivation of milk samples prior to screening, which is usual in bulk milk testing, is necessary particularly for the detection of residues of antimicrobial agents in individual milk samples. Testing of heat-treated samples protects the farmer against losses due to an exclusion from supply of milk yielding a false positive reaction.

A very detailed study of validation of methods for the detection of residues of antimicrobial agents in milk was published by Gardner et al. (1996). Its authors proposed a validation procedure for methods of assessment of individual milk samples which consists of the following four phases: 1. preliminary assessment of sensitivity and specificity; 2. determination of the detection limit; 3. assessment of factors that may cause false positive results; 4. testing under field conditions. For a system of monitoring of antimicrobial agents in milk, they proposed to use in the first phase a highly sensitive method for the initial screening and subsequently an inexpensive and highly specific confirmation method. Our paper is a contribution to the validation of methods used in the third phase of the above procedure.

The validation of screening tests for residues of antimicrobial agents described in this paper is beneficial particularly when testing of individual milk samples at the farm level is considered. In our opinion, such screening at the level of individual samples is an unavoidable step within each integrated monitoring system for residues of antimicrobial drugs. Information on tests suitable for the detection of residues should be provided by drug manufacturers.

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OSTEOCALCIN: A BIOCHEMICAL MARKER OF BONE FORMATION IN EQUINE MEDICINE

OSTEOKALCIN: BIOCHEMICKÝ MARKER TVORBY KOSTÍ V MEDICÍNĚ KONÍ

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ABSTRACT: Osteocalcin is the most abundant non-collagenous bone protein. It is produced by osteoblasts and is accepted as a biochemical marker of bone formation. The authors summarize the current knowledge of physicochemical properties, biosynthesis and functional aspects of osteocalcin in various species. In addition, determinants of plasma/serum osteocalcin concentration in the equine species are described. Factors affecting blood osteocalcin concentration recognized in horses are: age, type of horse, circadian rhythm, season, administration of exogenous corticosteroids and general anesthesia. In combination with osteocalcin measurements the kidney function should always be evaluated because it is the main way for osteocalcin clearance. Serum/plasma osteocalcin measurements are used in horses for training evaluation, for diagnostic purpose and to monitor the effect of a medical therapy on equine bone.

horse; osteocalcin; bone metabolism; bone formation

ABSTRAKT: Osteokalcin je nejhojněji se vyskytující nekolagenní kostní bílkovina. Tvoří se v osteoblastech a je chápán jako biochemický marker tvorby kosti. Je předložen přehled současných poznatků o fyzikálně chemických vlastnostech osteokalcinu, jeho biosyntéze a funkčních aspektech u různých druhů zvířat. Kromě toho jsou popsány určující faktory koncentrace osteokalcinu v krevní plazmě nebo séru. Tyto faktory ovlivňují koncentraci osteokalcinu v krvi koní: věk, typ koně, denní rytmus, roční období, podávání exogenních kortikosteroidů a celková anestezie. Spolu se zjišťováním koncentrace osteokalcinu by se vždy měla hodnotit funkce ledvin, protože se jedná o hlavní cestu vylučování osteokalcinu. Zjišťování koncentrace osteokalcinu v krevním séru nebo plazmě se používá k hodnocení fyzické kondice, k diagnostickým účelům a k monitorování vlivu léčebné terapie na kosti koní.

kůň; osteokalcin; kostní metabolismus; tvorba kostí

CONTENTS

OBSAH

1. INTRODUCTION

Fatigue failure of bone is the most common cause of musculoskeletal injuries in horses and is an important economic and welfare problem to the equine industry (Estberg et al., 1996). The assessment of bone metabolism and the early detection of bone diseases remain still a difficult problem in horses (Lepage, 1998).

Bone metabolism is characterized by two opposite but complementary activities: bone resorption and bone formation. The rate of bone turnover can be assessed by histomorphometry (Lepage et al., 1996) or by measuring the amount of specific biochemical bone markers, which are released into the bloodstream or into urine (Arnaud, 1996; Christenson, 1997). Bone resorption markers include urinary hydroxyproline, pyridinoline crosslinks, plasma telopeptide of type I collagen and tartrate-resistant acid phosphatase (TRAP). Bone formation markers include serum bone specific alkaline phosphatase (b-AP), propeptides of procollagen type I and osteocalcin (OC; Christenson, 1997).

Until a few years ago, blood measurements of total alkaline phosphatase (total AP) – an ectoenzyme located on the cell surface of osteoblasts – was the most widely used biochemical bone marker (Prockop et al., 1979). But total AP is not specific for bone formation because liver, gastrointestinal tract, placenta, certain tumors and perhaps other sources contribute to its concentration in the bloodstream. Bone specific alkaline phosphatase (b-AP) is much more bone specific than total AP. Nevertheless, b-AP can only be detected in horses in large amounts during the first year of life (Thorčn-Tolling, 1988). Research was performed to find a new bone formation marker in horses and this led to OC. Osteocalcin is a small, non-collagenous protein which is specific for bone tissue and dentin. It is accepted as a biochemical marker of bone formation (Ducy et al., 1996).

In this paper, general considerations for the use of OC and the current knowledge of OC in the equine species are described.

2. GENERAL CONSIDERATIONS

The name osteocalcin (*osteo* (Greek): for bone; *calc* (Latin): for lime salts; *in*: for protein) derives from Ca^{2+} affinity of this protein (Hauschka and Carr, 1982) and from the abundance of this protein in bone tissue (Hauschka et al., 1975; Lian et al., 1982; Conn and Termine, 1985). This protein was discovered in chicken (Hauschka et al., 1975) and bovine bone (Price et al., 1976). Osteocalcin is predominantly synthesized by osteoblasts and is then incorporated into the extracellular matrix of bone. A fraction of newly synthesized OC is released into the bloodstream, where it can be measured by radioimmunoassay (RIA; Delmas, 1990) or enzyme linked immuno-specific assay (ELISA; Hyldstrup et al., 1989). Osteocalcin has also been called the vitamin K-dependent protein of bone and bone gamma-carboxyglutamic acid protein or BGP (Price et al., 1976; Nishimoto and Price, 1979). The protein contains, depending on the species considered, three Gla residues at positions 17, 21 and 24 in the primary structure of 47–51 amino acid residues (Poser et al., 1980; Linde et al., 1980; Carr et al., 1981; Hauschka et al., 1982). Osteocalcin shows avid binding to solid calcium phosphate mineral surfaces, particularly in the case of hydroxyapatite crystals (Hauschka et al., 1975; Price et al., 1976; Poser and Price, 1979) and has a modest affinity for ionic calcium. The conformation of OC was described by a model of two antiparallel α-helical domains, connected by a peptide segment containing a β-turn (residues 26–29) and stabilized by Cys-23 – Cys-29 disulfide bond (Hauschka and Carr, 1982).

The biosynthesis of OC involves three vitamins: vitamin K for Gla residue formation (Hauschka and Reid, 1978), vitamin C for hydroxylation of prohydroxyproline into hydroxyproline (Hauschka et al., 1989) and vitamin D for stimulation of OC production (Price and Baukol, 1980). It was shown that the mouse genome contains an OC cluster composed of three genes (Rahman et al., 1993; Desbois et al., 1994). The first two, named OC gene 1 (OC1) and OC gene 2 (OC2), are expressed only in bone, while the third one, the OC related gene (ORG), is transcribed in the kidney, where a protein similar to OC has been described and called nefrocalcin (Desbois et al., 1994). Osteocalcin is a product of osteoblasts and odontoblasts (Camarda et al., 1987). The plasma/serum OC concentration correlates with new protein synthesis (Hauschka et al., 1989). Histomorphometric studies have shown that the absence of OC led to an increase in bone formation without impairing bone resorption (Ducy et al., 1996). A variety of other OC properties are resumed in Tab. I.

3. OSTEOCALCIN IN EQUINE MEDICINE

3.1. Sample collection and handling of osteocalcin

For accurate measurement of equine OC, samples are centrifuged within 90 minutes after venous blood collection. Serum/plasma are then stored at –20 °C in plastic tubes for up to 26 weeks (Hope et al., 1993). Serum may be thawed and refrozen up to 5 times without significant changes (Hope et al., 1993). The suggested optimum time for sampling would be daytime (light period) before exercise, since daytime fluctuations in serum OC concentrations occur within a narrow range (Lepage et al., 1991; Black et al., 1999). In addition, the effect of exercise is still not completely known in horses. Osteocalcin can be measured in equine serum or plasma, using a commercially available bovine RIA-Kit (Incstar Co, Stillwater Minn. USA; DSL, Webster TX, USA; Patterson-Allen et al., 1982; Lepage et al., 1990; Black et al., 1999). The human immunoradiometric assay (IRMA) does not recognize equine OC (Lepage et al., 1997). When interpreting the

I. Proposed functional properties of osteocalcin

Properties	References
Inhibitor of hydroxyapatite precipitation	Menanteau et al. (1982), Price (1985)
Informative molecule for $1,25(OH)_2D$	Pan and Price (1984), Skjodt et al. (1985)
Inhibitor of leukocyte elastase	Hauschka (1985)
Informative molecule for Ca^{2+} and Mg^{2+}	Hauschka and Carr (1982)
Activator/chemoattractant for cells with bone-resorbing properties	Malone et al. (1982), Mundy and Poser (1983), Chenu et al. (1994)
Chemoattractant for other cell types	Lucas et al. (1988), Lucas and Caplan (1988)

plasma/serum OC concentration in horses, creatinine and/or urea measurements have to be done for renal function evaluation (Lepage et al., 1990). This is based on studies in human medicine, where renal glomerular function impairment induces a variation in the circulating OC concentration (Cole et al., 1985).

3.2. Physiological determinants

3.2.1. Age

An inverse correlation between age and serum OC concentration was observed in horses with an important decrease of the OC level during the first 30 months of life (Lepage et al., 1990). Individuals less than one year of age have mean concentrations of serum OC of 47.3 ng/ml, individuals aged 1.5 to 2.5 years have 35.7 ng/ml and individuals over 3.5 years have a mean OC concentration of 6.7 ng/ml. These data indicate a significant slowdown in the rate of bone formation in adults, compared to foals (Lepage et al., 1990; Price et al., 1995; Black et al., 1999) and a predictive model for serum OC concentration based on 99 standardbred horses aged 0 to 5 years was established: $y = 52.19 - 0.026\,x$ age (x = age expressed in months; $r^2 = 0.76$; Lepage et al., 1992).

3.2.2. Gender

No significant influence of sex on serum OC concentration was observed (Lepage et al., 1992). The study was based on 52 male and 47 female standardbreds aged 36 to 60 months. However, two to three years old female thoroughbreds showed a significantly higher serum OC concentration than the same aged males (Chiappa et al., 1999). The authors explained this with the beginning of puberty in two to three years old thoroughbreds, which can cause a significant difference in OC levels. In another study, four months old quarter horse colts showed higher OC levels than the same aged fillies (Fletcher et al., 1998). This finding was explained by a faster growth rate in males when compared to females (Goyal et al., 1981).

Another study, treating the influence of general anesthesia on plasma OC concentration showed a difference between stallions and geldings/females (Grafenau et al., 1999). The decrease in plasma OC concentration after general anesthesia was significantly lower in stallions. This discrepancy was not explained and might be due to the small number of stallions in the above mentioned study ($n = 5$).

3.2.3. Type and breed of horses

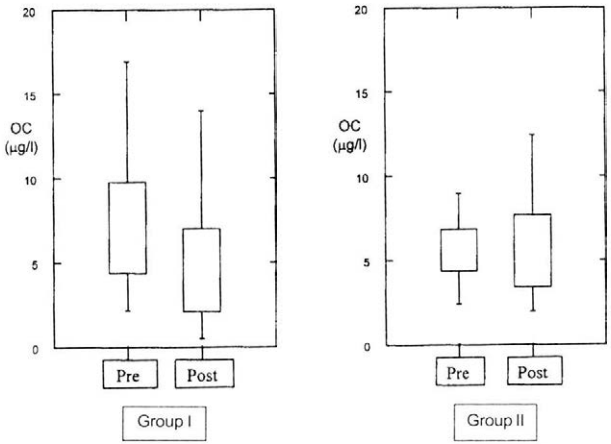
No significant difference was observed between four different breeds of horses (Lepage et al., 1998a). Nevertheless, when taking into account the horses' types, lower serum OC concentrations were observed in draught horses (adjusted least square mean (LSM) = 6.612 µg/l) compared to warmblood horses (LSM = 8.596 µg/l). Values for draught horses showed the smallest variation, resulting in a higher determination coefficient. A hereditary morphologic and physiologic characteristic of these horses might reflect a lower rate in bone remodeling in draught horses compared to warmblood horses.

3.2.4. Circadian rhythm

The circadian serum OC concentration showed a biphasic pattern in adult horses (Lepage et al., 1991). Levels were constant during daytime (light period) and showed significant variations during the night (dark period), going through a nadir at 8.00 p.m. and through a maximum peak at five o'clock in the morning. Another study indicated similar serum OC concentrations in adult geldings (Black et al., 1999), but not in weanlings. For the understanding of diurnal serum OC variations it is therefore inappropriate to include skeletally mature and immature animals in the same study group (Black et al., 1999). In contrast, a continuous lightening program made the circadian serum OC variations disappear in horses (Hope et al., 1993).

3.2.5. Season

Seasonal variations of serum OC concentration are observed in horses (Maenpää et al., 1988). Serum OC concentration decreased markedly in November and increased in December and January. These data were interpreted as an indication of substantial slowdown of bone formation after transfer from pasture to stables. One explanation given by the author of the study is a decrease in physical activity and a decrease in serum free thyroxin concentration. Other studies showed an increase in serum OC concentration in yearlings and two year old thoroughbred horses in spring-time (Jackson et al., 1998; Price et al., 1997).



1. Plasma osteocalcin concentration shortly before sedation (Pre) and 24 hours later (Post). A significant decrease is observed for the horses who had an anaesthesia (Group I) compared to the control group (Group II)

3.3. Effect of endogenous or exogenous corticosteroids

Endogenous cortisol has been measured in different studies (Hope et al., 1993; Geor et al., 1995; Grafenau et al., 1999). In none of these studies a correlation between OC and cortisol has been described. In addition, neither mean nor individual data demonstrated a consistent relationship between 24-h rhythms in serum cortisol and those of serum OC (Black et al., 1999).

In humans, a dose-dependent effect of exogenous corticosteroids on OC synthesis was documented *in vitro* (Beresford et al., 1984) and *in vivo* (Reid et al., 1986; Patterson-Buckendahl et al., 1988; Nielsen et al., 1988). In horses, serum OC concentration decreased within 24 to 48 hours after intramuscular administration of triamcinolone acetonide at a dosage of 0.09 mg/kg of body weight (Lepage et al., 1993). In seven out of ten horses of that study a return to pretreatment values was observed after 28 days. Pretreatment values for the other three horses were reached between 62 and 150 days later. Serum OC concentration also decreased markedly within 12 hours of intravenous, intramuscular or oral dexamethasone administration and returned to the pretreatment concentration within three days (Geor et al., 1995). The decrease of serum OC concentration in glucocorticoid-treated horses reflects reduction in the rate of bone formation. Therefore, serum OC concentration may be an early sensitive index of the deleterious effect of long-term corticosteroid therapy on bone metabolism in the equine species.

3.4. Influence of anesthesia and surgery

General anesthesia and surgery were associated with some metabolic and hormonal changes in horses (Robertson et al., 1987; 1990). Plasma OC concentration decreased significantly within 24 hours after general anesthesia (Fig. 1) performed with or without a surgical procedure (Grafenau et al., 1999). The duration of anesthesia had a significant influence on the degree of plasma OC concentration decrease. Plasma OC concentration increased slowly within the first 24 hours after anesthesia and reached normal values four days after. The described decrease of serum OC after an orthopedic procedure in a foal (Lepage and Marcoux, 1991) should be attributed to the effect of general anesthesia and probably not to the surgical therapy.

3.5. Influence of physical training

One of the fundamental elements to get horses with strong bones is a correct exercise. Indeed the bone tries to change its shape when getting strains. This adaptation to strains is performed by modeling and remodeling activities. The main problem in horses is to find a sensitive way to individually monitor exercise based on objective parameters. Therefore studies were performed to detect the influence of exercise on biochemical bone markers.

The effect of treadmill exercise on the cortex of the third metacarpal bone of young horses has been described (Buckingham and Jeffcott, 1991; McCarthy and Jeffcott, 1992). In that study histomorphometric evaluation of the dorsal cortical bone showed extensive modeling in an exercised group of horses and it was shown that the serum OC concentration was lower after 14 weeks of exercise compared to horses of the unexercised group. These results are in accordance with another study, where two years old thoroughbred mares were divided in a treadmill-exercised group and a control group. A significantly lower serum OC concentration was observed in the exercised group within the first month compared to a control group (Jackson et al., 1998).

In another study stall housed Arabian yearlings showed a lower serum OC concentration at day 14 compared to a pasture held control group. Following day 14 the serum OC concentration of the stalled horses returned to baseline. The authors attribute these variations to a transient slowdown in the rate of osteoblastic activity (Hoekstra et al., 1999). A recent study also revealed that three months deconditioning did not affect serum OC concentrations (Porr et al., 1998).

3.6. Influence of feeding

The effect on serum OC concentration of a forced exercise (longeing) combined or not with oral glucosamine supplementation has been studied (Fenton et al., 1999). Exercised horses had lower concentrations of serum OC compared to not exercised horses. Not exercised, supplemented horses tended to have higher serum OC levels, when compared to exercised non-supplemented and exercised supplemented horses (Fenton et al., 1999). In another study, foals fed a low-copper diet had higher serum OC levels as their withers height increased, compared to a control group (Hurtig et al., 1991). Finally it has been shown that highly exercised Arabian horses taken out of training and fed either a low calcium diet or a high calcium diet during a three months deconditioning period had no influence on their serum OC concentration (Porr et al., 1998).

3.7. Influence of bone diseases

Serum OC concentration increased slightly in horses with developmental orthopedic diseases (Hurtig et al., 1991). Copper-restricted foals may have had continuing stimulation of osteoblasts after their period of fastest growth due to increased modeling of defective bone (Hurtig et al., 1991).

A comparison between variations of serum OC and AP levels in a four months old foal before resection of a wide exostosis on the third metacarpus showed higher serum OC concentrations before surgery (107 ng/ml) and 18 days post-operatively (215 ng/ml; Lepage and Marcoux, 1991). But total AP concentrations were in the normal range of values during the whole time. The results for serum OC were compared with the mean values of eight same aged healthy foals (59 ± 13 ng/ml). A 16 months old Paint horse yearling presented with generalized dyschondroplasia and vertebral epiphysiolysis, showed serum OC levels (17.9 ng/ml) of about half the mean serum OC level of age-matched individuals (Girard et al., 1997). Serum OC levels slightly below normal (31.88 ng/ml) were observed in a seven months old foal which developed a juvenile mandibular ossifying fibroma (Lepage and Davis, 1992). This decrease was strongly pronounced during the first days after bilateral mandibulectomy (15.44 ng/ml). We interpreted the decrease by a stress response due to surgery. Indeed, we showed in another study that corticosteroids diminished serum OC in horses (Lepage et al., 1993). Horses with chronic obstructive pulmonary disease showed normal serum OC concentrations.

3.8. Future for bone markers in horses

In horses, an evaluation of fracture risk, a response to medical treatment of the skeletron and training-monitoring will be possible, if veterinarians are able to interpret bone mineral density changes or other skeletal parameters reflecting the bone metabolism (Ducy et al., 1996). A simple blood sample will then allow the veterinarian to have more accuracy in predicting and evaluating bone pathologies and will also monitor the response of equine bone to physical training or to a medical treatment.

Osteocalcin is reflecting bone formation and since each biochemical marker reflects a different physiological process in bone, it would enhance the diagnostic power, if we could select a group of markers. This was already observed in a previous study with the concomitant use of serum ICTP and serum OC to evaluate bone metabolism in draught and warmblood horses (Lepage et al., 1998a).

But it should always be remembered that biochemical bone markers do not provide information about skeletal mass, elasticity or architecture, which are better evaluated by densitometric techniques like quantitative ultrasonography (Lepage et al., 1998b), scintigraphy (Tucker et al., 1998) or photon absorptiometry (Jeffcott et al., 1988).

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