Slovak J. Anim. Sci., 42, 2009, SUPPLEMENT 1: 104-109

ISSN 1337-9984

Scientific paper formerly presented

EFFECT OF SUBCHRONIC EXPOSURE TO TOLYLFLUANID ON THE LACTATE DEHYDROGENASE ACTIVITY AND ITS ISOENZYMES IN SHEEP

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ABSTRACT

Tolylfluanid (1,1-dichloro-N-[dimethylamino)-sulfonyl]-1-fluoro-N-(4-methylphenyl) methanesulphenamide is a foliar basic fungicide with protective action and additional acaricidal effects. In this study we have evaluated sub-chronic effects of tolylfluanid, administered at a dose of 93 mg.kg^{-1} b.wt (1/20 LD_{50}) for 7 days/week for a period of 28, 60 and 90 days on the activities of LDH and its isoenzymes in the blood plasma and haemolyzates of sheep. The data showed no significant differences in total activity of LDH (μkat.l^{-1}) and its isoenzymes (%) in the blood plasma of the control and experimental animals examined before the experiment and after 28-, 60- and 90-day-exposure. In the experimental group of sheep significant changes of LDH-1 isoenzyme (P=0.046) and LDH-2 isoenzyme (P=0.011) activities in haemolyzates after 28- and 90-day exposure to tolylfluanid have been registered.

Key words: sheep; lactate dehydrogenase; isoenzymes; tolylfluanid; blood plasma; haemolyzates

INTRODUCTION

A release of natural or synthetic substances is one of the important factors in the degradation of the biosphere by human activities. Pesticides together with heavy metals from emissions are dominant compounds of the chemical load of the environment of man and animals (Kramárová et al., 2005; Monteiro et al., 2006; Çadlar and Kolankaya 2008). Main problems are resulted in clinical and subclinical effect leading to losses in animal performance or in residue contamination of animal products which may later be consumed by humans (Cerón et al., 1995). Tolylfluanid (1,1-dichloro-N-[dimethylamino)-sulfonyl]-1-fluoro-N-(4-methylphenyl) methanesulphenamide is a foliar basic fungicide with a protective action and additional acaricidal effects. It is used in deciduous fruit crops, grapes, ornamentals and vegetables. In animals tolylfluanid is rapidly absorbed and hydrolyzed within 48 h to form dimethylamino sulphotoluidite (DMST) and then transformed to the main metabolite 4-(dimethylamino sulfonyllamino) benzoic acid, which can be further dimethylated. There is no accumulation in organs and tissues (Abbink and Weber, 1988). Sub-chronic and chronic toxicity studies conducted in animals showed altered liver enzyme activities, thyroid hormone levels and increased incidence of hyperplastic and neoplastic lesions of the thyroid gland and damage of renal cortical tubules (PFPC Newsletter Special Issue, 1999; Kovalkovičová et al., 2008).

The use of biomarkers has been proposed to evaluate the effects of pollutants, such as heavy metals and organic xenobiotic compounds. Biomarkers give a measure of exposure to toxic effects or of susceptibility (Fossi, 1994). The use of biochemical reactions in biological

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assessment of the impact of chemical substances on the environment increased in past years. At sub-individual levels biochemical markers have been suggested as indicators of chemical exposure and different effects (Mishra and Shukla, 1997; Šalplachta and Vinkler, 2001; Šutiaková et al., 2004). Mammalian LDHs [(l) -lactate: NAD^+ oxidoreductase, EC 1.1.1.27] are tetrameric NAD^+-specific dehydrogenases that catalyze the interconversion of lactate and pyruvate and participate in both glucose catabolism and gluconeogenesis from lactate. Somatic cells differentially express five combinations of tetramers derived from ldh-a and ldh-b, which are named LDH-1, LDH-2, LDH-3, LDH-4 and LDH-5 (Yu et al., 2001). Alterations in the LDH isoenzyme spectrum of tissues induced by toxic conditions reflect a metabolic cellular dysfunction of these tissues (Ribeiro et al., 1999; Arai et al., 2003; Tripathi and Verma, 2004).

Our previous results suggested that the exposure to Euparen Multi (tolylfluanid) may cause genome damage in somatic cells (Šutiaková et al., 2006). According to Velisek et al. (2006) assessment of the ecotoxicological risks caused by pesticides to ecosystems is based on the data on toxicity and effects of pesticide preparations to non-target organisms. A study of the effect of pesticides on the condition of multiple molecular forms of enzymes makes it possible to judge not only the effect of the preparation on a given enzyme, but also to make assumptions concerning its effect on biochemical processes, which determine causal-evidential relationships between molecular mechanisms, which serve as the basis of the toxic action of pesticides.

In this study we have evaluated sub-chronic effects of tolylfluanid which was administered at a dose of 93 $mg.kg^{-1}$ b.wt (1/20 LD_{50}) for 7 days/week for a period of 28, 60 and 90 days on the activities of LDH and its isoenzymes in the blood plasma and haemolyzates of sheep.

MATERIALS AND METHODS

The organisation of the experiment, the investigations conducted and the related documentation complied with legislative regulations governing the protection of experimental animals of the Slovak Republic.

Animals

The experiment was carried out on 12 clinically healthy 18-24-month-old female sheep (Merino breed), which have not been previously exposed to pesticides or any other agents suspected of being toxic. There were seven sheep weighing 50.79 ± 6.52 kg in the experimental group. The control group consisted of 5 sheep with a mean b.wt. 49.10 ± 4.98 kg. Animals were housed, treated with the anthelminthic agent Aldifal 2.5 % susp. a.u.v. (Mevak a. s., Nitra, SR) at a dose of 2 ml per 10 kg b.wt., acclimatized for 1 week before dosing and observed before an initiation of the study to ensure that they were healthy. Only animals found to be in a clinically acceptable condition were assigned to the study. During the study a food and water were offered *ad libitum*. Animals were housed at 8-12 °C; 70 % relative humidity. Food consumption, general condition and any other clinical symptoms were monitored daily. The reference values for pulse and breath frequency, temperature and rumen rotations were in a good agreement with those described by Slanina et al. (1993). Body weights were recorded weekly.

Tested pesticide

Seven experimental animals were exposed to Euparen Multi manufactured by Bayer A. G., containing 50 % of tolylfluanid (1,1-dichloro-N-[dimethylamino)-sulfonyl]-1-fluoro-N-(4-methylphenyl).

Dose and exposure

The tested pesticide preparation was freshly prepared each day and administered by a rumen sonde at a dose of 93 $mg.kg^{-1}$ b.wt. (46.5 mg active ingredient. kg^{-1} b.wt.; 1/20 LD_{50}) daily for 28, 60 and 90 days. The dose was calculated on the basis of mean LD_{50} value of tolylfluanid for sheep and its purity, as reported by Hoffmann (1983); i.e. 937.5 ± 312.5 $mg.kg^{-1}$ b.wt. (purity 99.2 %).

Collection of blood samples

The blood was taken from the jugular vein (*vena jugularis*) into tubes with heparin (15 $IU.ml^{-1}$) in the morning, before the experiment on Day 0 and on Day 28, 60 and 90 after the start of the experiment (in the set time intervals). Blood samples were centrifuged for 20 min at 190 g and the blood plasma was measured within 12 h.

Preparation of haemolyzates

Haemolyzates were prepared from venous blood samples collected into the heparinized tubes. Before analysis the red cells were washed several times with a Ringer′s solution following the same procedure as described previously (Beutler et al., 1977).

Enzyme activity measurement

The total LDH activity was determined spectrophotometrically using the lactate as the substrate, according to Setrove and Makarova (1989). The measurements were made in duplicates.

Electrophoresis

LDH isoenzymes were separated electrophoretically on a buffered polyacrylamide gel at pH 8.3 according to Dietz and Lubrano (1967). The isoenzyme bands were scanned by a densitometer model DS 90 at 525 nm. Values for each isoenzyme were calculated by a computer and expressed as a percentage of the enzyme activity.

Statistical analysis

The data were analysed using the statistical software (Sigma-Stat® Jandel Scientific). All values are expressed at the mean ± SD and the differences between means were analysed by ANOVA - test.

RESULTS

Table 1 represents the results of total LDH (μkat. l^{-1}) and its isoenzymes (%) in the blood plasma of control and experimental animals. Administration of tolylfluanid caused no significant differences in total activities of this enzyme. No significant differences in the activities of LDH isoenzymes were observed in the experimental group of sheep after the exposure to tolylfluanid in the blood plasma. The activities of the LDH biomarker isoenzyme in haemolyzates of control and experimental animals are shown in Table 2. There was a significant increase in the activities of LDH-1 isoenzyme (P=0.046) and LDH-2 isoenzyme (P=0.011) in sheep after 28-, resp. 90-day exposure to tolylfluanid when compared to control.

Table 1: The effect of tolylfluanid on the LDH activities and on its isoenzymes in the blood plasma

Sampling/day	Groups of animals	Statistical values	Total activities (μkat.l^{-1})	Isoenzymes of LDH in the blood plasma (%)				
				LDH1	LDH2	LDH3	LDH4	LDH5
0 Day	Control group (n=5)	Mean	3.35	84.28	2.86	4.47	0.85	7.02
		± SD	0.64	10.17	1.15	1.00	1.13	10.33
	Experim. group (n=7)	Mean	3.53	89.86	3.55	5.67	0.54	0.38
		± SD	0.42	3.50	1.40	0.74	0.64	0.34
		P	0.546	0.202	0.389	0199	0.537	0.091
Exposure 28 Days	Control group (n=5)	Mean	2.91	94.23	1.14	2.17	0.67	1.79
		± SD	0.49	1.71	0.16	1.52	0.37	1.03
	Experim. group (n=7)	Mean	3.27	94.09	1.33	1.55	0.59	2.45
		± SD	0.59	1.28	0.84	0.79	0.45	1.32
		P	0.296	0.882	0.631	0.370	0.733	0.382
Exposure 60 Days	Control group (n=5)	Mean	3.68	89.04	3.44	5.62	0.79	1.11
		± SD	0.41	2.68	1.59	1.39	0.47	1.24
	Experim. group (n=7)	Mean	3.96	84.02	5.27	8.93	0.97	0.82
		± SD	0.49	4.80	2.24	3.42	0.89	0.87
		P	0.339	0.068	0.161	0.074	0.701	0.655
Exposure 90 Days	Control group (n=5)	Mean	2.62	71.92	5.53	20.04	1.31	1.00
		± SD	0.18	6.61	1.36	5.08	1.28	0.05
	Experim. group (n=7)	Mean	3.17	69.74	6.76	19.78	1.17	2.55
		± SD	0.66	6.71	1.61	4.85	1.31	3.37
		P	0.104	0.602	0.211	0.933	0.824	0.402

n - number of animals analyzed; x - arithmetic mean; SD - standard deviation; tot. act. - total activity; sample./day. - sampling day; exp. -exposure

Table 2: Comparison of the activities of isoenzymes LDH of sheep haemolyzates before and after exposure to tolylfluanid

Sampling/day	Groups of animals	Statistical values	Isoenzymes of LDH in the blood plasma (%)				
			LDH1	LDH2	LDH3	LDH4	LDH5
Day/0	Control group (n=5)	Mean	93.96	1.85	0.84	0.83	2.51
		± SD	0.87	0.70	0.27	0.56	0.70
	Experim. group (n=7)	Mean	92.86	2.44	1.86	0.71	2.13
		± SD	2.59	1.23	1.15	0.51	1.22
		P	0.387	0.362	0.085	0.716	0.551
Exposure Days/28	Control group (n=5)	Mean	95.85	1.74	1.06	0.59	0.81
		± SD	2.07	1.15	0.76	0.41	0.51
	Experim. group (n=7)	Mean	97.80*	0.97	0.42	0.23	0.58
		± SD	0.84	0.69	0.45	0.22	0.32
		P	0.046	0.174	0.123	0.07	0.366
Exposure Days/60	Control group (n=5)	Mean	84.29	1.61	2.04	2.69	9.37
		± SD	9.98	0.54	1.46	2.56	9.57
	Experim. group (n=7)	Mean	83.61	1.36	5.76	2.80	6.48
		± SD	10.62	0.55	8.85	2.12	5.31
		P	0.915	0.466	0.381	0.943	0.541
Exposure Days/90	Control group (n=5)	Mean	90.48	2.03	1.37	2.26	3.86
		± SD	2.17	0.69	0.49	0.91	1.34
	Experim. group (n=7)	Mean	84.37	4.00**	6.43	2.03	2.97
		± SD	6.95	1.21	5.08	0.93	0.23
		P	0.103	0.011	0.055	0.697	0.140

Explanation: *P= 0.046 ** P= 0.011

DISCUSSION

Activity of enzymes in the blood plasma can also be used as a relevant stress indicator. LDH is an important glycolytic enzyme which is present in the cells of almost all body tissues and changes in the enzyme activity may provide direct and indirect evidence of the cellular damage and can indicate the toxic mechanism. LDH is a terminal enzyme of anaerobic glycolysis, therefore, being of crucial importance to the muscular physiology, particularly in conditions of chemical stress, when high levels of energy may be required in a short period of time (Baghi et al., 1995; Young et al., 1999; De Coen et al., 2001). Biochemical and molecular parameters that are specific and sensitive may be useful for identifying doses below which increases in biomarkers are not statistically significant (Andersen and Barton, 1998). Our results suggest no significant release of this enzyme from the tissue cytoplasm into the blood plasma. LDH is a highly sensitive but not specific biomarker. The function of specific indicators is performed by LDH isoenzymes which allow us to identify the damage either to tissues or a least to organs (e.g. action of bacterial toxins, some xenobiotics, etc.) (Gupta et al., 1991; Šalplachta and

Vinkler, 2001). Specific enzymes regulating a variety of metabolic pathways can be altered, as a result of stress-related homeostatic adjustments induced by toxicant exposure. Also, there are some interspecies differences in the activity of LDH isoenzymes, which can be affected by physiological factors, such as age, sex, etc. (Bláhovec et al., 1992; Avallone et al., 1996).

Mammalian red blood cells are enucleated cells that normally circulate for several months; therefore its cell function is supported through the primitive and universal pathways. The glycolytic pathway is the only pathway of ATP synthesis in the mature cell which is well adapted to its role in oxygen transport (Fujii and Miwa, 1999). The difference of the distribution of LDH isoenzymes in tissues is known to reflect differences in their metabolic activity. The myocardial tissue and erythrocytes contain a preponderance of anode migrating LDH-1 and LDH-2, which mainly consist of H subunit. A total activity of LDH in sheep of red blood cells was low (Beathy and Doxey, 1983), because they determined only LDH isoenzyme patterns. Alterations in the normal erythrocytary LDH zymogram are expressive of a changed red cell population. This is suggestive of a hyperactive erythropoid system. Such a condition, in fact, may be induced by xenobiotics exposures. We determined a significant increase in the activities of LDH-1 isoenzyme and LDH-2 isoenzyme in sheep after 28 and 90 days of exposure to tolylfluanid. The variation of total LDH activity in a population of cells from an individual is large, but relative activities of the isoenzymes LDH-1 and LDH-2 are fairly constant. This can be explained by a distribution of cell age in the population (Xue and Yeung, 1994).

This study suggests that alterations in the isoenzyme LDH activities may provide insight into the site of toxic injury and reveal which tissues or organs are involved. Further studies are necessary in this research because the presence of xenobiotics as pesticides may affect the health and production of animals.

CONCLUSION

The results of our study showed that lactate dehydrogenase isoenzyme activities in the blood plasma and in haemolyzates may serve as a useful quantitative *in vivo* biomarker for ecotoxicological studies in animals.

ACKNOWLEDGEMENT

The project was supported by National Reference Laboratory for Pesticides, University of Veterinary Medicine, Košice, VEGA Grants No.1/4375/07 and 1/0545/08, Slovak Republic.

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