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## THE EFFECT OF BISPHENOL A ON SHEEP LACTATE DEHYDROGENASE ENZYME SYSTEM *IN VITRO*

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### ABSTRACT

Lactate dehydrogenase (LDH) and its isoenzymes should serve both as the indicators of cell membrane integrity, as well as cell viability one, *in vitro*. Our experiments and observations of the cytotoxic effects of bisphenol A (BPA) in the culture medium of sheep (48 treatment time) were carried out at the concentrations of  $1 \times 10^{-4}$ ,  $1 \times 10^{-5}$ ,  $1 \times 10^{-6}$  and  $1 \times 10^{-7}$  mol.L<sup>-1</sup>. LDH and its isozymes of the culture medium were used as an endpoint for cytotoxicity studies. The Anova tested data of our experiments showed no significant differences in the both total activity of LDH ( $\mu\text{kat.L}^{-1}$ ) and also its isoenzymes ( $\mu\text{kat.L}^{-1}$ ) in the culture medium of our control and experimental group after 48 exposure to BPA. Our results showed that LDH should serve as potential marker of cell injury.

**Keywords:** *in vitro*, culture medium, sheep, lactate dehydrogenase, bisphenol A, cytotoxicity

Bisphenol A (BPA), 2, 2,-bis (4-hydroxyphenyl) propane, is an industrial chemical that is made by combining acetone and phenol. It is extensively used as a monomer in the production of polycarbonate plastics and as a precursor of epoxy resins. Polycarbonate (PC) is widely used in the manufacture of food containers (e.g., milk, water and infant bottles) and epoxy resins are used as an interior protective lining for food and beverage cans (**Aschberger et al., 2010**). BPA is studied mainly in relation to human and animal health. The presence of BPA in the environment can cause serious health problems (endocrine disruptions, neurotoxic, genotoxic and other problems) (**Wu et al., 2013; Renz et al., 2013; Loganathan and Kannan, 2011**). **Yan et al., (2013)** investigated the acute effects of BPA on ventricular arrhythmias and infarction in rat hearts. Authors stated that in addition to endogenous pathophysiological conditions, have also important role the environmental chemicals in these diseases.

There are different methodological approaches in toxicological research to identify hazards and to verify the processes which influence the expression of response of living organism to pesticides, different xenobiotics and food-associated chemicals (**Eisenbrand et al., 2002**). The damages caused by xenobiotics depend on their bioavailability, metabolism and on other factors (e.g. as is tissue turnover rate etc.). At low and intermediate concentration of xenobiotics they may impair specific functions. In contrast at high concentration of agents they cause general cytotoxicity and cell death (**Camatini et al., 1996**). *In vitro* toxicological studies are very important and relevant endpoints. These endpoints have been established in many cell types which determine, for example the membrane permeability changes. Intracellular lactate dehydrogenase (EC. 1.1.1.27), L-(+)-lactate: NAD<sup>+</sup> oxidoreductase (LDH) leakage is a well known indicator of cell membrane integrity and cell viability (**Legrand et al., 1992**). LDH catalyses the intraconversion of pyruvate and lactate and is involved in both the catabolism and anabolism of carbohydrates. In addition to metabolic roles in cells includes in a number of other biological processes (**Powers et al., 1991**). In animals LDH was found to be a tetrameric molecule, which exists in five different isoenzymes LDH 1 – LDH 5. There are at least two gene duplication events which have led to three genetically subunits. LDH-A or M

(muscle)-type, LDH-B or H (heart)-type and LDH-C or X-type are known in vertebrates and each species has different physico-chemical properties, biological function and development regulations (**Kopperschläger and Kirchberger, 1996**).

The present studies comprised *in vitro* analyses of the potential cytotoxic effect of BPA in the blood of sheep on the basis of LDH and isoenzymes, released into culture medium.

## MATERIALS AND METHODS

### Chemicals – culture medium

BPA, synonyms: [2, 2-Bis (4-hydroxyphenyl) propane], 4, 4'-Isopropylidene-diphenol, with formula  $(\text{CH}_3)_2\text{C}(\text{C}_6\text{H}_4\text{OH})_2$ , molecular weight 228.29, CAS No. 80-05-7 (Sigma-Aldrich, USA). BPA was dissolved in dimethylsulfoxide (DMSO) and was freshly prepared before each experiment at concentrations of:  $1 \times 10^{-4}$ ,  $1 \times 10^{-5}$ ,  $1 \times 10^{-6}$  and  $1 \times 10^{-7}$  mol.L<sup>-1</sup>. The final concentration of DMSO in the cultures was 0.5%. The tested substances were added at 24 h after culture initiation and left until the end of cultivation (48 h treatment time). Cytochalasin-B (Cyt-B, CAS No. 14930-96-2), mytomycin C (MMC, CAS No. 50-07-7), DMSO, (CAS No. 67-68-5), and penicillin G (benzyl penicillin sodium salt; CAS No. 200-710-2) were obtained from Sigma, USA. Streptomycin (streptomycin sulphate salt; CAS No. 3810-74-0) was obtained from Sigma-Aldrich, USA. Cyt-B was dissolved in DMSO. MMC at a final concentration of 0.25 µg.mL<sup>-1</sup> was dissolved in distilled water and served as the positive control to ensure the validity of the assay. Chromosome medium serum-free Panserine 701, CAS No. P0-710701 M, and 7.5% NaHCO<sub>3</sub> were obtained from PAN Systems GmbH, Biotechnologische Produkte, Germany. Heparin (heparinum natricum) was obtained from Zentiva, a. s., Czech Republic.

### Assay of Total LDH activity

The concentration of total LDH (µkat.L<sup>-1</sup>) activity was assessed using commercial diagnostic kits (Randox, United Kingdom) with automatic biochemical analyser Alizé (Lisabio, France). Before determination we mixed 300 µl of reagents (phosphate buffer with concentration 50 mmol.L<sup>-1</sup> and pH 7.5; pyruvate with concentration 0.6 mmol.L<sup>-1</sup>; NADH with concentration 0.18 mmol.L<sup>-1</sup>) with 5 µl of sample and measuring absorbance at 340 nm.

### LDH isoenzyme activity

For electrophoretic study, 10 µl of culture supernatant was used for each separation. Hydrasys device (Sebia, France) was used for the determination of LDH isoenzymes activity (µkat. L<sup>-1</sup>). The samples were separated using commercial electrophoretic kits Hydragel 7 ISO-LDH (Ecomed, Žilina) on alkaline buffered (pH 8.4) agarose gels. Each gel contained agarose (0.8g/dL) and alkaline buffer (pH 8.40±0.05). Substrate contained phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (NAD) and nitro blue tetrazolium (NBT). Blocking solution contained acetic acid (5%) and citric acid (0.5%). For determination apply 10 µl of serum into the applicators, and then place the applicators into the wet storage chamber for 5 minutes. Put the gel plate into the device and start the migration at 20 °C for 8 minutes. Apply substrate solution (45 °C/ 20 minutes) and cool (20 °C/ 5 minutes). Remove the remaining substrate solution and pipette the blocking solution with incubation at 20 °C for 10 minutes. Remove the blocking solution and apply one thick filter paper and dry. The dried gels were prepared for visual examination and densitometry to obtain accurate relative quantification of individual zones. Then photographs of the gels were taken. Qualitative evaluations of the gels were done directly from the electrophoretograms and the densitometric curves of the separations were created by means of Epson Perfection V 700 Photo densitometer scanning at 570 nm. The visualization of LDH isoenzymes was performed according to the following reactions:

#### LDH

Lactate + NAD → Pyruvate + NADH<sub>2</sub>

NADH<sub>2</sub> + PMS → Reduced PMS + NAD

Reduced PMS + NBT → PMS + Reduced NBT (precipitate of Formosan-blue)

The amount of resulting Formosan precipitate was proportional to the LDH enzymatic activity.

### Statistical Analysis

The Sigma Stat program (Statistical software™, Jandel Scientific) was employed for statistical evaluation of the results. The mean frequency of activity LDH cells and its isoenzyme were calculated based on the results of two independent experiments, and the statistically significant differences between control and treatment values were determined using analysis of variance (ANOVA) at the 95% or higher confidence level.

### RESULTS

Table 1 shows the results of total LDH ( $\mu\text{kat.L}^{-1}$ ) and its isoenzymes ( $\mu\text{kat.L}^{-1}$ ) in the supernatant of the culture medium. The exposure of BPA caused no significant differences in total activities of this enzyme at the given concentrations. No significant differences in the activities of LDH isoenzymes were observed in the experimental group in the supernatant of the culture medium after the exposure to BPA.

Table 1.

#### The effect of bisphenol A on the LDH activities and on its isoenzymes of sheep in culture supernatants.

Tested substance/ Groups	Treatment period (h) Dose( $\text{M.L}^{-1}$ )	Statistical values	Total activities of LDH [ $\mu\text{kat.L}^{-1}$ ]	Isoenzymes of LDH in sheep cultural supernatants [ $\mu\text{kat.L}^{-1}$ ]				
				LDH 1	LDH 2	LDH 3	LDH 4	LDH 5
DMSO, (Neg. control),	48 h	$\emptyset$	2.76	1.33	0.23	0.83	0.17	0.21
		$\pm\text{SD}$	1.32	0.48	0.16	0.52	0.09	0.08
		SEM	0.93	0.34	0.12	0.37	0.06	0.06
Experiments, BPA	48 h $1 \times 10^{-4}$	$\emptyset$	2.26	1.23	0.15	0.60	0.12	0.18
		$\pm\text{SD}$	0.47	0.23	0.04	0.52	0.14	0.14
		SEM	0.33	0.17	0.03	0.37	0.01	0.01
		P	0.84	0.91	0.60	0.60	0.50	0.70
	48 h $1 \times 10^{-5}$	$\emptyset$	1.93	0.99	0.15	0.03	0.13	0.18
		$\pm\text{SD}$	0.20	0.10	0.05	0.09	0.00	0.04
		SEM	0.14	0.87	0.04	0.07	0.00	0.03
		P	0.47	0.60	0.57	0.79	0.57	0.70
	48 h $1 \times 10^{-6}$	$\emptyset$	1.94	0.99	0.13	0.55	0.09	0.19
		$\pm\text{SD}$	0.30	0.11	0.05	0.08	0.04	0.04
		SEM	0.22	0.08	0.04	0.06	0.03	0.03
		P	0.48	0.42	0.50	0.53	0.36	0.8
48 h $1 \times 10^{-7}$	$\emptyset$	2.16	1.10	0.16	0.60	0.14	0.16	
	$\pm\text{SD}$	1.07	0.42	0.12	0.30	0.09	0.14	
	SEM	0.76	0.30	0.09	0.22	0.06	0.10	
	P	0.66	0.65	0.67	0.64	0.71	0.73	

Comments: LDH = Lactate dehydrogenase;  $\emptyset$  = Mean values;  $\pm\text{SD}$  = Standard deviation; SEM = Standard error mean; P = Statistical significance of results; h = hours, DMSO = Dimethylsulphoxide, BPA = Bisphenol A.

### DISCUSSION

The *in vitro* viability test of the animal cell culture is used for determination the possible hazards of xenobiotics. One of the advantages of LDH activity determination in the supernatants of cell

cultures is based on the fact that the examination reveals changes in cell viability and not in cell metabolism (Racher *et al.*, 1990). Our results suggest no significant release of this enzyme from the ovine blood cells into the culture medium. The assumptions are made that intracellular enzymes are only released after damage to the cell membrane and all activity is released from damaged cells into culture medium (Goergen *et al.*, 1993). We observed that at the concentrations of BPA decreased total activity of LDH. But on the other hand there are also other parameters that may influence the intracellular activity of LDH from other cells, as well as the pH, chemical composition of the medium and some other factors (Geaguey *et al.*, 1990). Kending and Tarloff, (2007) used LDH as an endpoint for cytotoxicity studies induced by several chemicals. Their studies suggest that LDH is released to the culture medium, and is susceptible to degradation when reactive chemicals are present. In our case, in the culture media were present such chemicals as is cytochalasin and also antibiotics. We simultaneously studied also the cytotoxic effect of BPA by calculating nuclear division index. In the case of nuclear division index BPA did not affect also cytotoxic index at all treatment concentrations (Šutiaková *et al.*, 2013). It is known that LDH is a highly sensitive but not specific marker. The function of specific indicators is performed by LDH isoenzymes, which allow us to identify the damage either to cells and tissues by different xenobiotics. The examination intracellular isoenzyme patterns allow also detect the interspecies cross-contamination in culture medium (Steube *et al.*, 1995; Nims *et al.*, 1998). However our results did not record significant differences between control and experimental groups at the different concentrations of bisphenol A in the medium. Biochemical and molecular parameters that are specific and sensitive may be useful for identifying their influences in biosystems through certain biomarkers also in situations when they are not statistically significant (Anderson and Barton, 1998).

Further studies are necessary in this research because the presence of such xenobiotics as is BPA may affect the health and production of animals and health of human.

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