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Original article

Immunohistochemical evaluation of superoxide dismutase (Cu/Zn SOD) concentrations in erythrocytes of dairy cattle and farm-raised deer by a computer-assisted analysis of microscopic images

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Abstract

The effectiveness of the immunohistochemical method in determining Cu/Zn SOD concentrations in red blood cells of dairy cattle and farm-raised deer was evaluated by a computer-assisted analysis of microscopic images and scanning technique. Superoxide dismutase (Cu/Zn SOD) concentrations in erythrocytes were determined in smears of whole blood samples collected from 16 Polish Holstein-Friesian cows and 22 farm-raised deer in spring. Mouse anti-bovine SOD (Cu-Zn) monoclonal antibodies (2F5, Serotec) were used in 1:50 dilution. The degree of immunostaining for SOD in red blood cells was determined with the use of the MIDI 3DHistech Panoramic Scanner (Hungary) and 3DHistech Panoramic Viewer, NuclearQuant and MembraneQuant software. Our findings indicate that the immunohistochemical method is a useful technique for evaluating Cu/Zn SOD concentrations in red blood cells of cattle and deer.

Key words: superoxide dismutase, erythrocytes, cattle, deer, immunohistochemical evaluation

Introduction

The results of recent research demonstrate that intensive dairy cattle production leads to homeostatic disorders, including disruptions of the prooxidant-antioxidant balance (Heidarpour et al. 2013,

Wang et al. 2013). Those disorders are caused by excessive production of reactive oxygen species and weakening of antioxidant mechanisms which rely on primary antioxidants, such as superoxide dismutase (SOD) and glutathione peroxidase (GSH), secondary antioxidants, including vitamins E and C, uric acid,

bilirubin and albumins, and tertiary antioxidants comprising DNA repair enzymes (Bartosz and Bartosz 1999, Bernabucci et al. 2005, Castillo et al. 2005). Prooxidant-antioxidant balance should be regularly monitored in high-yielding dairy cows. Special attention should be given to antioxidant protection which can be disrupted in various production phases. In mammals, there are three families of superoxide dismutase, an antioxidant enzyme. Superoxide dismutase with the Cu/Zn cofactor is found inside cells in the cytoplasm. Superoxide dismutase containing manganese atoms is also found inside cells, mainly in the mitochondria. The third type of superoxide dismutase also contains copper and zinc, but it is found outside cells in the blood plasma (Frei et al. 1988, Andrzejak et al. 1995, Bartosz and Bartosz 1999, Droge 2002). The results of our previous research and other authors' findings suggest that SOD is a reliable indicator of subclinical diseases, in particular disorders caused by deteriorating health of high-yielding dairy cows in the perinatal period, unbalanced diet, subclinical ketosis and mineral deficiencies (Bernabucci et al. 2005, Barszcz et al. 2009).

In humans and animals, superoxide dismutase activity in whole blood and plasma is generally determined by indirect methods based on SOD's ability to inhibit other reactions that involve superoxide anion radicals O_2^- . In this approach, the degree of SOD-inhibited cytochrome C reduction is determined by measuring the increase in absorbance at 550 nm wavelength.

SOD activity is most commonly measured by the Ransod method which employs xanthine and xanthine oxidase to generate superoxide radicals and form a stain. This method allows the determination of SOD activity in hemolysate, whole blood and blood plasma. The Ransod method has been long applied in our laboratory for research and analytical purposes. It is an accurate analytical technique, but determinations of SOD activity have to be performed in completely hemolyzed blood samples (Beauchamp and Fridovich 1971).

This study evaluates the effectiveness of the immunohistochemical method for determining SOD concentrations in erythrocytes in blood smears on microscope slides. Whole blood smears of cattle and deer blood and anti-bovine SOD monoclonal antibodies were used in the experiment. SOD concentrations in red blood cells were evaluated by computer-assisted image analysis.

Materials and Methods

Blood sampling

Superoxide dismutase (Cu/Zn SOD) activity in erythrocytes was evaluated in smears of whole blood sampled from 16 Polish Holstein-Friesian cows during

a dry period (43-60 days before parturition) and 22 farm-raised deer in spring. All animals were clinically healthy, and their hematological and biochemical parameters of peripheral blood were within the reference ranges. The average red blood cell counts were determined at $6.37 \times 10^{12}/l$ (from 5.22 to $7.02 \times 10^{12}/l$) in dairy cows and $11.38 \times 10^{12}/l$ (from 9.49 to $13.6 \times 10^{12}/l$) in deer. In the morning, blood was collected from the external jugular vein into chilled tubes containing potassium versenate. Immediately after sampling, 5 μ l of blood from each animal was smeared on two microscope slides in accordance with the standard procedure.

Immunohistochemical determination of SOD in blood smears

Air-dried smears were fixed with Bio-Fix (Bio-Optica) and left to dry. The fixing agent was sprayed evenly on specimens to produce a thin layer of film on the slide. Endogenous peroxide was blocked with the Peroxidase Block System (EnVision+ System – HRP/DAB, DAKO). The primary antibody was applied. Specimens were incubated with the mouse anti-bovine SOD (Cu/Zn) monoclonal primary antibody (Serotec) diluted 1:50 (Primary Antibody Diluent, Serotec) in a wet chamber for 60 minutes. The primary antibody-antigen interaction was visualized with the use of secondary antibodies conjugated with horseradish peroxidase and diaminobenzidine substrate (EnVision+ System – HRP/DAB for use with primary mouse antibodies, DAKO). The primary antibody was not applied in negative control, and it was incubated in diluent alone. A positive reaction was reported when red blood cells incubated with the mouse anti-bovine SOD (Cu/Zn) antibody formed a brown stain.

Computer-assisted image analysis

The above activity levels were adopted to evaluate all red blood cells in all analyzed smears. Three blood smears from every animal were scanned using the 3DHitech MIDI Panoramic Scanner (Hungary). Staining intensity was evaluated using the Nuclear-Quant software (Hungary). Three pathologists set the intensity levels independently, and then the consensus 0-3 point scale was prepared (0= none, 1=weak, 2=medium and 3=strong). Once set, all the smears were subjected to automated analysis. Six randomly chosen areas of each smear (100 000-110 000 μ m²) were evaluated. The results were expressed as a percentage of erythrocytes showing none, weak, medium and strong reactivity, respectively.

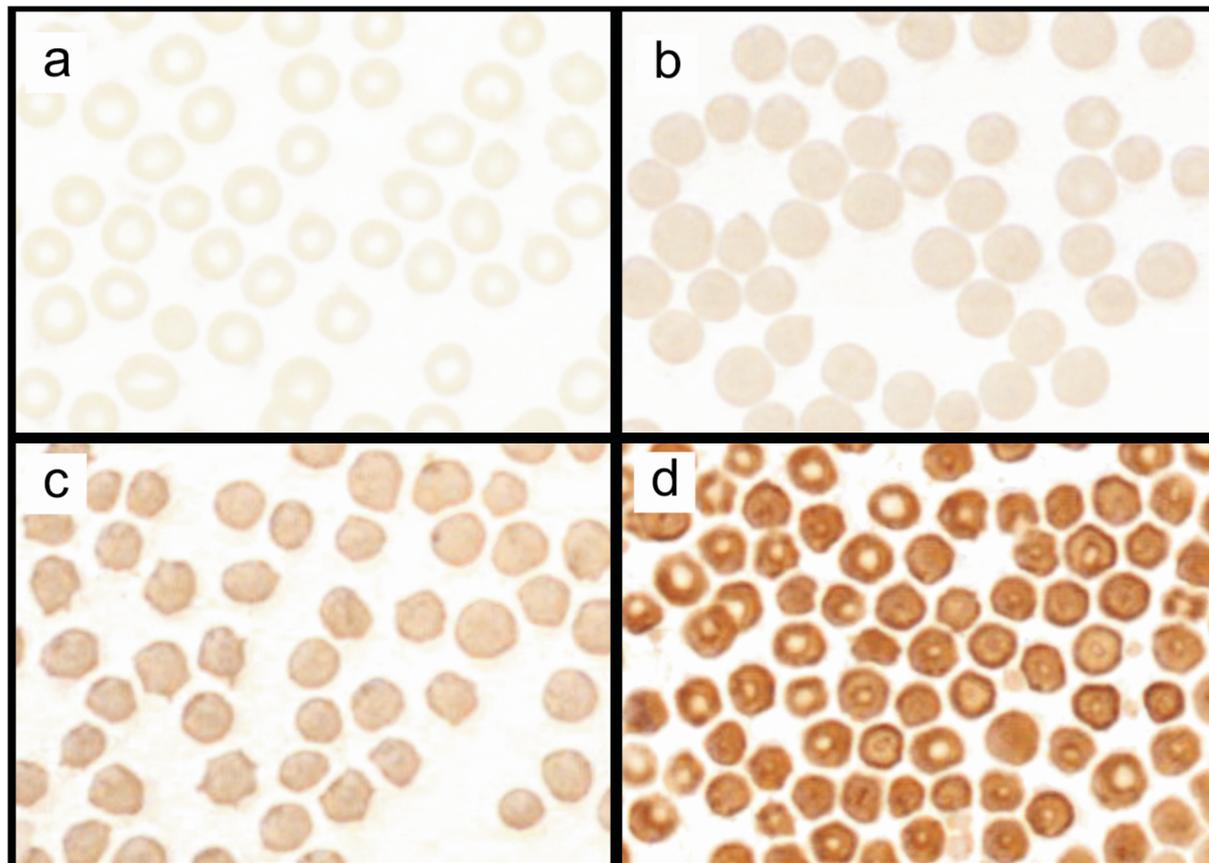


Fig. 1. Variations in immunostaining for Cu/Zn SOD in red blood cells of dairy cows: a – negative (-), b – weak (+), c – medium (++), d – very strong (+++). Magnification 720x.

The statistical analysis was conducted using software SPSS 19.0. The relationship between the groups' scores was estimated by Friedman test and Wilcoxon test. Differences were considered significant when $p < 0,05$.

Results

Red blood cells with different SOD concentrations in dairy cattle smears are presented in Figures 1(a-d). The differences in SOD levels are manifested by varied intensity of brown staining. Red blood cells from deer smears incubated with anti-bovine SOD (Cu/Zn) monoclonal antibodies produced similar images (Fig. 2 a-d). The average SOD (Cu/Zn) concentrations determined in red blood cells of cattle and farm-raised deer during computer-assisted image analysis are presented in Table 1.

Discussion

The results of the immunohistochemical evaluation of deer erythrocytes suggest that anti-bovine

SOD (Cu/Zn) monoclonal antibodies can also be used to determine SOD concentrations in the red blood cells of farm-raised deer. Analyses of three successive smears from different animals revealed similar SOD concentrations in red blood cells, and the observed differences resulted from cell overlaps in smears and were not statistically significant. To date, SOD concentrations were most often evaluated by the immunohistochemical method in tissue samples, and various morphometric techniques were deployed to evaluate positive reactions (Change et al. 1988).

Our results suggest that clinically healthy ruminants are characterized by significant variations in erythrocyte SOD levels. In dry dairy cows, 95% of the examined red blood cells showed very strong immunostaining (+++) for Cu/Zn SOD. In an on-going study of a larger dairy cattle population of various breeds and at different production phases Kołodziejaska (author's unpublished data, 2013), showed significant changes observed in erythrocyte SOD concentrations evaluated with the use of the immunohistochemical method.

The analysis of red blood cells of farm-raised deer revealed more significant variations in Cu/Zn SOD concentrations. Very high SOD levels (+++) were

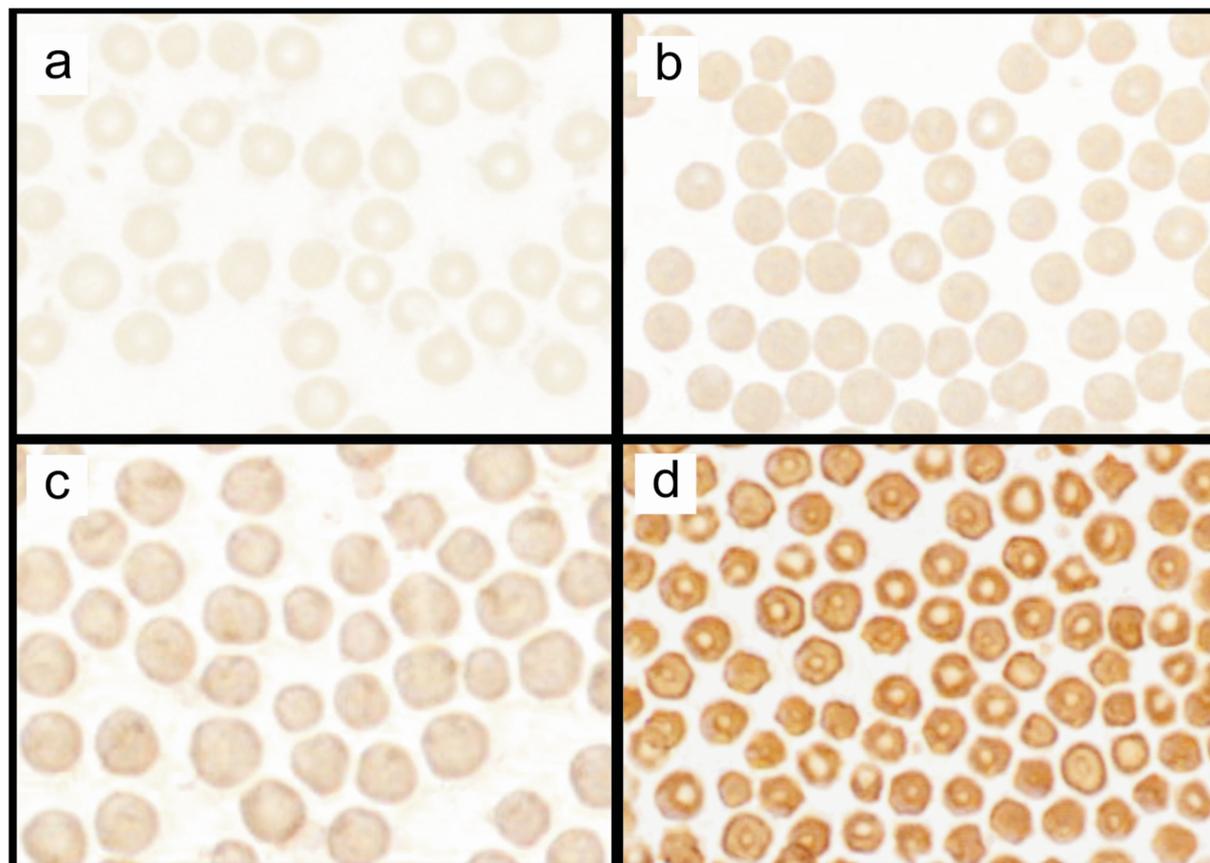


Fig. 2. Variations in immunostaining for Cu/Zn SOD in red blood cells of farm-raised deer: a – negative (-), b – weak (+), c – medium (++) , d – very strong (+++). Magnification 720x.

Table 1. RBC percentages and counts in cows and deer with different levels of immunostaining for Cu/Zn SOD. Mean values (X) and SE were determined in a computer-assisted analysis of blood smear images.

Species	Population	Total RBC counts $10^{12}/l$	RBC percentages and counts with different levels of immunostaining for Cu/Zn SOD		
			Degree	% RBC	RBC counts $10^{12}/l$
Cows	16	6.38±0.15	-	0	0
			+	0.76±0.30	0.048±0.019
			++	4.00±1.01	0.255±0.067
			+++	95.24±1.08	6.082±0.145
Deer	22	11.36±0.43	-	0	0
			+	23.51±5.76	2.68±0.721
			++	21.40±3.37	2.43±0.388
			+++	55.09±7.39	6.25±0.891

Key: (-) no immunostaining, (+) weak immunostaining, (++) medium immunostaining, (+++) very strong immunostaining.

observed in more than 50% of the analyzed erythrocytes, whereas weak (+) and medium (++) immunostaining for Cu/Zn SOD was reported in approximately 20% of the studied cells, respectively.

The results of this study indicate that the immunohistochemical method supports the determination of SOD concentrations in red blood cells with the use of anti-bovine SOD monoclonal antibodies. The

immunohistochemical technique contributes new knowledge and has important practical implications for computer-assisted analyses of microscopic images. Our findings demonstrate that anti-bovine SOD monoclonal antibodies can be effectively used in analyses of deer erythrocytes.

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