Immunoistochemical 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,
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 6.37x10^{12}/l (from 5.22 to 7.02x10^{12}/l) in dairy cows and 11.38x10^{12}/l (from 9.49 to 13.6x10^{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 μl 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 3DHistech 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 μm^2) 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 Kołodziejska (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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Population</th>
<th>Total RBC counts $10^{12}$/l</th>
<th>RBC percentages and counts with different levels of immunostaining for Cu/Zn SOD</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Degree</td>
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<tr>
<td>Cows</td>
<td>16</td>
<td>6.38±0.15</td>
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<tr>
<td>Deer</td>
<td>22</td>
<td>11.36±0.43</td>
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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.

Acknowledgements

This study was supported by research grant N.N. 308562840

References