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References

Effect of Prolonged Storage on the Activities of Superoxide Dismutase, Glutathione Reductase, and Glutathione Peroxidase, Clifford Aliaba,1* Farida Al-Awadi,2 and Samuel Olusi3 (1 Department of Medical Laboratory Science, Faculty of Allied Health Sciences, Kuwait University, P.O. Box 31470, Kuwait; Departments of 2 Biochemistry and 3 Pathology, Faculty of Medicine, Kuwait University, Kuwait; * author for correspondence: fax 965-4830937, e-mail clifford@hsc.kuniv.edu.kw)

Reactive oxygen species such as superoxide and hydrogen peroxide react in human tissues to form hydroxyl free radicals, especially when catalyzed by transition metals, e.g., iron [Fe(II)] and copper [Cu(I)]. The product is highly electrophilic and damaging to surrounding tissues and is implicated in the pathology of debilitating human diseases such as atherosclerosis (1), Parkinson disease and other neurodegenerative disorders (2), and cancer (3). Copper zinc-superoxide dismutase (CuZn-SOD; EC 1.15.1.1), glutathione peroxidase (GPX; EC 1.11.1.9), and glutathione reductase (GR; EC 1.6.4.2) are cellular antioxidants that can protect cells from the potentially harmful effects of reactive oxygen species (4–6). Assays of the activities of these mainly intracellular enzymes form part of the indirect determination of the activity of free radicals.

Measurements of erythrocyte CuZn-SOD, GPX, and GR activities usually are performed in the assessment of antioxidant status, and there has been speculation on the stability of these enzymes over a long period of storage. In this study, we investigated the long-term stability of CuZn-SOD, GPX, and GR in washed erythrocyte hemolysates by comparing the activities in stored hemolysates with those in hemolysates from freshly drawn blood. Subjects were recruited from blood donors (n = 24) with the consent of the ethics committee. The blood donors fulfilled the health requirements of the blood transfusion center, i.e., absence of contagious disease, medication, or history of viral hepatitis, malaria, and drug addiction. Venous blood (5 mL) was collected from each donor into a potassium-EDTA Vacutainer Tube by means of a cannula inserted into the arm of the recumbent donor. The blood was centrifuged within 4 h of sampling at 1620g for 10 min in a refrigerated centrifuge at 4°C to separate the plasma. The Buffy coat was removed, and the remaining erythrocytes were drawn from the bottom, washed three times in cold saline (9.0 g/L NaCl), and hemolyzed by the addition of an equal volume of ice-cold demineralized ultrapure water (MilliQ plus reagent grade; Millipore) to yield a 50% hemolysate. Aliquots of the hemolysates (500 μL) were prepared for immediate assay of CuZn-SOD, GPX, and GR. Additional aliquots (500 μL) of the remaining hemolysates were dispensed into 1.5-mL capacity Beckman microfuge tubes (Beckman Instruments), capped, and frozen at −80°C; these aliquots were assayed for CuZn-SOD, GPX, and GR after storage for 5, 12, and 21 months. To the 50% hemolysates (500 μL), was added 1 mL of demineralized water; 10 μL of each diluted hemolysate was brought to a final volume of 1.25 mL with Ransod sample diluent (a solution of 0.01 mol/L phosphate buffer, pH 7.0), thus bringing the final dilution of the hemolysates assayed for the activity of CuZn-SOD to 1.500. This dilution gives a 40–60% inhibition by hemolysate CuZn-SOD of the generation of superoxide anion free radical (O₂·) by the reagent xanthine and xanthine oxidase (XOD).

Measurement of CuZn-SOD activity was performed using Ransod reagents (Randox Laboratories) and is based on the method developed by McCord and Fridovich (7) coupling O₂· generators (xanthine and XOD) with an O₂· detector [2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride]. Absorbance was monitored in a Beckman DU 7500 spectrophotometer (Beckman Instru-
ments) for 30 s (for the initial reading, \( A_1 \)) after the addition of XOD (125 \( \mu \)L) as the starting reagent, and subsequently for 3 min for the final reading (\( A_2 \)). The final reaction volume was 1 mL. All rates for the calibrators and diluted samples were converted into percentages of the rate for the sample diluent (uninhibited) and subtracted from 100% to give a percentage of inhibition. The unit of activity of the assay is defined as the amount of CuZn-SOD that inhibits the rate of formazan dye formation by 50%.

Measurement of GPX activity was performed using Ransel reagents (Randox) and is based on the method of Paglia and Valentine (8). GPX catalyzes the oxidation of reduced glutathione (GSH) by cumene hydroperoxide. In the presence of GR and NADPH, the oxidized glutathione (GSG) is immediately converted to the reduced form (GSH) with concomitant oxidation of NADPH to NADP\(^+\). The decrease in absorbance at 340 nm is measured. Drabkin cyanide-ferricyanide solution, pH 7.0–7.4, was prepared by dissolving potassium ferricyanide (200 mg), potassium cyanide (50 mg), and potassium dihydrogen phosphate (140 mg) in distilled water, making the volume up to 1 liter, and mixing after the addition of 0.5 mL of 250 g/L Brij 35.

For the GPX assay, hemolysates (50 \( \mu \)L) were diluted with 1 mL of Ransel diluting agent (Ransel reagent; 1:40). The dilution of the hemolysates before assay, 1:82). The diluting agent reduces any GSGS present in the hemolysates to GSH because the cyanide in the Drabkin reagent rapidly inactivates GSGS. The cyanide in the Drabkin reagent inhibits other peroxidases that may be present in human blood and prevents falsely high results.

GR activity was assayed using reagent from Randox Laboratories; the assay was adapted from the method of Goldberg and Spooner (9). GR catalyzes the reduction of GSSG in the presence of NADPH, which is oxidized to NADP\(^+\). The decrease in absorbance at 340 nm is measured.

For the GR assay, the 50% hemolysates were centrifuged to remove stroma, and 100 \( \mu \)L was diluted with 1.9 mL of 9 g/L NaCl (total dilution of the hemolysates for the GR assay, 1:40).

Statistical analyses were performed using SPSS 9.0 for Window software (SPSS). Differences in the means between groups were analyzed by one-way ANOVA. Two-tailed \( P \) values were used, and statistical significance was at \( P < 0.05 \).

The activities of erythrocyte CuZn-SOD, GPX, and GR (mean \( \pm \) SE, in kU/L) obtained for the fresh hemolysates were compared with the activities of enzymes in hemolysates after storage at \(-80 ^\circ \)C for 6, 12, and 21 months (Table 1). There were no significant changes in the activities of the enzymes between fresh and frozen hemolysates.

Previous studies generally compared the activities of cytoprotective enzymes for groups of patients with matched controls. The observed differences in enzyme activities in most of these studies could constitute a contributory cause of the disease, an effect of drug treatment, drug overdose, or xenobiotics. However, we are unaware of any published study on the stability of these enzymes in washed human erythrocytes. The uncertainty about their stability often necessitated the assay of the cytoprotective enzymes shortly after hemolysate preparation. We have demonstrated that CuZn-SOD, GPX, and GR in washed erythrocyte hemolysates are stable at \(-80 ^\circ \)C for close to 2 years. We believe that our observations will be useful in large-scale epidemiological studies involving cytoprotective enzymes by providing the knowledge that these enzymes are stable over a prolonged period at \(-80 ^\circ \)C.

### References

### Table 1. Comparison of the activities of SOD, GPX, and GR in fresh and frozen hemolysates (n = 24).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fresh</th>
<th>6 months</th>
<th>12 months</th>
<th>21 months</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD, kU/L</td>
<td>270 ± 8</td>
<td>270 ± 8</td>
<td>271 ± 8</td>
<td>267 ± 7</td>
<td>0.982</td>
</tr>
<tr>
<td>GPX, kU/L</td>
<td>10.9 ± 0.6</td>
<td>10.7 ± 0.6</td>
<td>10.9 ± 0.7</td>
<td>10.8 ± 0.7</td>
<td>0.996</td>
</tr>
<tr>
<td>GR, kU/L</td>
<td>1.26 ± 0.09</td>
<td>1.28 ± 0.09</td>
<td>1.27 ± 0.09</td>
<td>1.24 ± 0.08</td>
<td>0.990</td>
</tr>
</tbody>
</table>

* Mean \( \pm \) SE.

### Response to Acute Osteoclast Activity Inhibition Assessed by the Determination of C-Telopeptide of Type I Collagen in Serum, *Isabella Villa, Barbara Saccon, and Alessandro Rubinacci* (Bone Metabolic Unit, Scientific Institute H San Raffaele, Via Olgettina 60, 20132 Milan, Italy; *author for correspondence: e-mail a.rubinacci@hsr.it*)

Several degradation products of bone matrix are released in serum by osteoclasts when they resorb bone. The most critical molecular fragments of type I collagen that have clinical utility as sensitive and specific markers of bone resorption contain the nonreducible pyridinium cross-link, deoxypyridinoline (Dpd), which is excreted in the urine in free and peptide-bound forms (1). Immunoassays