Supplemental Digital Content 1: Methods

Methods for the Determination of Erythrocyte Metabolites, Erythrocyte Enzymatic Activities and Plasma Free Hemoglobin, Antioxidant Power, and Lactate Dehydrogenase

Citrate anticoagulated blood was sampled from the femoral artery. adenosine triphosphate, pyruvate, lactate and 2,3 diphosphoglycerate, were determined in an aliquot of blood immediately processed according to Beutler[1]. Briefly, blood was deproteinized by ice cold perchloric acid (patient-conrolled analgesia, 3% final concentration), centrifuged (10.000 g, 5 min at 4°C) and the supernatant neutralized with a solution of potassium carbonate. The solution was centrifuged to remove the white precipitate and the supernatant used for the biochemical determinations. Adenosine triphosphate and pyruvate were determined according to Beutler[1], lactate by the BioVision assay kit (Milpitas, CA) and 2,3 diphosphoglycerate by a Roche Diagnostics kit (Mannheim Germany).

In parallel, another aliquot of blood was briefly centrifuged (1,800 g, 10 min) and plasma was evaluated for the presence of free hemoglobin as marker of hemolysis, by reading the absorbance at 412 nm (Soret Band) and for the total antioxidant power (Power of AntiOxidant), based on the scavenging ability of plasma antioxidants to the long-life radical monocation 2,2'-azinobis (3-ethylbenzothiazoline-6-suslfonic acid (Antioxidant Assay kit , Sigma, St. Louis, MO).

Erythrocytes were washed twice and then resuspended in approximately one volume of saline, hemolysed by dilution (1:10 vol/vol) with the stabilizing solution (0,005% mercaptoethanol-0,1% EDTA) and used for the analysis of erythrocyte enzymes. Glyceraldehyde phosphate dehydrogenase, lactate dehydrogenase, and acetyl cholinesterase were measured according to Beutler[1], catalase and glutathione reductase according to Aebi *et al*[2] and Pinto *et al*[3] respectively. Levels of metabolic intermediates, antioxidant power and enzymatic activities were evaluated at each experimental time in three aliquots of blood processed separately and referred (nmoles or U) to the hemoglobin content. This allows to exclude the differences due to the dilution of plasma at 24 and 48 h. Meta-hemoglobin was estimated from visible spectra of blood hemolysate with the algorithm of Winterbourn[4] based on the optical densities at 560, 577 and 630 nm.

Methods for Matrix Metalloproteinases Analysis

For the analysis of matrix metalloproteinases activity, samples were frozen and stored at -80°C. Tissues were homogenized in 10 mM Tris-HCl, 150 mM NaCl, 20 mM EDTA, pH 7.5, and the protein content was assessed using the Bradford method. The same amount of protein in each extract (5µg) was loaded onto sodium dodecyl sulfate polyacrylamide gels containing 1 mg/ml gelatin, and the samples were run at 150 V for 1 h in a minigel apparatus. The samples were loaded into the gels without heat denaturation and reducing agents. After the run, the gels were washed at room temperature for 2 h in 2.5% Triton X-100 and incubated overnight at 37°C in 10 mM CaCl2, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.5 buffer. The gel was stained in 2% (v/v) Coomassie Blue G-250 in fixing solution and photographed on a light box after appropriate destaining. Proteolysis was detected as white bands in a dark blue field[5-7]. The gels were than scanned and imaged in black and white, and gelatinolytic bands were quantified using ImageJ

software (National Institutes of Health, Bethesda, MD) and Adobe Photoshop (Adobe Software, Seattle, WA). Densities were expressed as arbitrary units. The examination was performed by a pathologist blinded to study groups.

References

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