Oxygen binding capacity

The oxygen binding/carrying capacity of polyethylene glycolated (PEGylated)-carboxyhemoglobin was determined at 37°C under normal atmosphere similarly to the methodology described elsewhere.[1,2] Briefly, PEGylated-carboxyhemoglobin (40 $mg.ml^{-1}$) was diluted in its own buffer (pH 7.38 - 7.40) for a final concentration of 4.0 $mg.ml^{-1}$. A fiber optic oxygen micro sensor using luminescence lifetime (MiniTip, Oxy Micro, World Precision Instruments, FL, USA) was used for the determination of oxygen concentration and was calibrated by a simple two-point calibration, 100% air-saturation and 0% air saturation (dithionite) with barometric pressure and temperature adjustment according to the manufacturer's recommendations. The fiber optic was placed in a double jacked thermostated (37°C) small cuvette through a dedicated hole (side) and sealed with a rubber. Two milliliters and a half (2.5 ml) of the solution were placed in the cuvette, continuously stirred with a magnetic bar and allowed to equilibrate to room air before being hermetically sealed. The concentration of oxygen ($\mu mol.l^{-1}$) in the medium was continuously recorded. After stabilization of the oxygen concentration in the cuvette, 10 µL $K_3Fe(CN)_6$ 1M (kept at 37°C in a thermostated bath and equilibrated at room air) was added through the rubber with a precise syringe. The release of bound-oxygen due to the conversion of oxy- to met-Hb was monitored.

The oxygen binding/carrying capacity was calculated as follows:

$$\Delta n_{O_2 released} = n_{O_2\ initial} - n_{O_2\ final} = C_{O_2\ initial}\ x\ V_{initial} - C_{O_2\ final}\ x\ V_{final} \qquad \textbf{\textit{Eq. 1}}$$

where n is the number of moles of O_2 (mol), C is the concentration of O_2 provided by the fiber optic sensor (mol.L-1) and V is the volume in the cuvette before (initial) and after (final) adding $K_3Fe(CN)_6$.

$$V_{O_2} = \Delta n_{O_2 released} \, x \, V_m \qquad \textbf{Eq. 2}$$

where V_{O_2} (L) represents the volume of oxygen released in the cuvette and V_m the molar volume (L.mol^{-1}) which is 25.45 L.mol^{-1} at 37°C (310.15°K) and under 1 atm (ideal gas law).

$$Oxygen\ content\ concentration = \frac{V_{O_2}}{V_{final}} \, x \, 10^3 \qquad \textbf{Eq. 3}$$

where the oxygen content concentration (ml O_2.L^{-1}) represents the volume of oxygen dissolved in the cuvette

$$Measured\ oxyen\ carrying\ capacity = \frac{oxygen\ content\ concentration}{C_{Hb\ final}} \qquad \textbf{Eq. 4}$$

where oxygen carrying capacity is expressed in mL O_2.g^{-1} Hb and $C_{Hb\ final}$ is the final concentration of hemoglobin/HBOC (g.L^{-1}) in the cuvette.

The difference between the solubility coefficients of oxygen in PEGylated-carboxyhemoglobin +buffer and $K_3Fe(CN)_6$ has been neglected.

Spectrophotometric analysis of PEGylated-carboxyhemoglobin and derivatives

The observed total absorption spectrum of each plasma sample was then compared to the known spectra of the different derivatives of bovine hemoglobin with their absorptivities.[3] With the use of the classical least squares method (mixture of 4 components and at 150 wavelengths), the different concentrations of each component were recovered. We used the methodology proposed by Prof. Tom O'Haver (https://terpconnect.umd.edu/~toh/spectrum), to recover the concentration of each component after entering the 4 known spectra of bovine hemoglobin: oxy-Hb, deoxy-Hb, met-Hb and carboxy-Hb. However, the met-Hb could not be accurately

determined due to a possible interference with Pd-TCCP. The original spectra of PEGylated-carboxyhemoglobin were provided by Prolong Pharmaceuticals and were found to perfectly match with the bovine hemoglobin spectra found in the publication of Zijlstra *et al.*[3]

The oxygen saturation of PEGylated-carboxyhemoglobin was determined as follows:

$$S_{\text{PEGylated-carboxyhemoglobin}}O_2(\%) = \frac{C_{oxy-\text{PEGylated-carboxyhemoglobin}}}{(C_{oxy-\text{PEGylated-carboxyhemoglobin}} + C_{deoxy-\text{PEGylated-carboxyhemoglobin}})}$$

Immunohistochemical analysis and histology

Kidney sections (4 µm) were deparaffinized with xylene and rehydrated with decreasing percentages of ethanol and finally with water. Antigen retrieval was accomplished by microwaving slides in citrate buffer (pH 6.0) (Thermo Scientific, AP-9003-500) for 10 min. Slides were left to cool for 20 min at room temperature and then rinsed with distilled water. Surroundings of the sections were marked with a PAP pen. The endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min at room temperature and later rinsed with distilled water and PBS. Blocking reagent (Lab Vision, TA-125-UB) was applied to each slide followed by 10 min incubation at room temperature in a humid chamber. Kidney sections were incubated for overnight at 4°C with rabbit polyclonal Tumor Necrosis Factor-α (1:100) (abcam 6671) and incubated for 1 h at room temperature with Lipocalin 2 antibody (Neutrophil Gelatinase-Associated Lipocalin, 1/150) (abcam 41105). Antibodies were diluted in a large volume of UltrAb Diluent (Thermo Scientific, TA-125-UD). The sections were washed in PBS three times for 5 min each time and then incubated for 30 min at room temperature with biotinylated goat anti-rabbit antibodies (LabVision, TP-125-BN). After slides were washed in PBS, the streptavidin peroxidase label reagent (LabVision, TS-125-HR) was applied for 30 min at room temperature in a humid chamber. The colored product was developed by incubation with AEC.

The slides were counterstained with Mayer's hematoxylin (LabVision, TA-125-MH) and mounted in vision mount (LabVision, TA-060-UG) after being washed in distilled water. Both the intensity and the distribution of specific Tumor Necrosis Factor-α and Neutrophil Gelatinase-Associated Lipocalin staining were scored. For each sample, a histological score (H-Score) value was derived by summing the percentages of cells that stained at each intensity multiplied by the weighted intensity of the staining [H-Score = S Pi (i+1), where i is the intensity score and Pi is the corresponding percentage of the cells] (Birman et al, 2012) under a light microscope at x400 magnification. Kidney sections were photographed using Leica Qwin microscope.

The kidney sections were stained with periodic acid-schiff reagent (PAS) and hematoxyline. Histologic changes in the cortex were assessed by quantitative measurements of tissue damage. Tubular damage was defined as loss of brush border, vacuolar degeneration, cast formation, and invagination. The degree of kidney damage was estimated at 400x magnification using 10 randomly selected fields for each animal by the following criteria: 0, normal; 1, areas of damage <10% of tubules; 2, damage involving 10% to 25% of tubules; 3, damage involving 25% to 50% of tubules; 4, damage involving 50% to 75% of tubules; 5, damage more than 75% of tubules. A minimum of 40 tubules were examined per group, with n=6/kidney per animal minimum.

References

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