Free Radical Biology and Medicine 124 (2018) 299–310

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original article

High- and low-affinity PEGylated hemoglobin-based oxygen carriers: Differential oxidative stress in a Guinea pig transfusion model

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ARTICLE INFO

Keywords:
Hemoglobin-based oxygen carriers
Blood substitutes
Oxidative stress
8-oxo-2'-deoxyguanosine
4-hydroxynonenal
Malondialdehyde
Oxygen affinity

ABSTRACT

Hemoglobin-based oxygen carriers (HBOCs) are an investigational replacement for blood transfusions and are known to cause oxidative damage to tissues. To investigate the correlation between their oxygen binding properties and these detrimental effects, we investigated two PEGylated HBOCs endowed with different oxygen binding properties - but otherwise chemically identical - in a Guinea pig transfusion model. Plasma samples were analyzed for biochemical markers of inflammation, tissue damage and organ dysfunction; proteins and lipids of heart and kidney extracts were analyzed for markers of oxidative damage. Overall, both HBOCs produced higher oxidative stress in comparison to an auto-transfusion control group. Particularly, tissue 4-hydroxynonenal adducts, tissue malondialdehyde adducts and plasma 8-oxo-2'-deoxyguanosine exhibited significantly higher levels in comparison with the control group. For malondialdehyde adducts, a higher level in the renal tissue was observed for animals treated with the high-affinity HBOC, hinting at a correlation between the HBOCs oxygen binding properties and the oxidative stress they produce. Moreover, we found that the high-affinity HBOC produced greater tissue oxygenation in comparison with the low affinity one, possibly correlating with the higher oxidative stress it induced.

1. Introduction

Hemoglobin-based oxygen carriers (HBOCs) have been investigated for more than 40 years as possible substitutes for whole blood transfusions. HBOCs are based on cell-free hemoglobin (Hb) that has undergone either chemical or genetic modifications to increase its retention in the bloodstream while maintaining the capability to deliver oxygen to tissues [1–4]. They include Hbs conjugated with bulky polymers [5–9], cross-linked and/or polymerized Hbs [10–12], and encapsulated Hbs [13–16]. HBOCs exhibit a wide range of biochemical and biophysical properties [17].

Conjugation of purified Hb with polyethylene glycol (PEG) has emerged as a particularly promising approach for the design of HBOCs [5–9,18–27]. However, PEGylation of Hb under aerobic conditions significantly alters its structural and functional properties, promoting tetramer dissociation, increasing oxygen affinity and abolishing oxygen binding cooperativity [27–29]. Indeed, the aerobically PEGylated Hb known as MP4, Hemospan or MP4OX (Sangart, San Diego, CA, USA), the first of these products to reach phase 2 clinical trials, exhibited a p50 close to that of R-state Hb, with minimal cooperativity in oxygen binding [5,22,30–32], mostly a consequence of Hb dimerization [28,33,34]. PEGylation with the same chemistry but under anaerobic

Abbreviations: 8-OHdG, 8-hydroxy-2'-deoxyguanosine; HbA, purified human hemoglobin A; BSA, bovine serum albumin; DNPH, 2,4-dinitrophenylhydrazine; EDTA, ethylenediamine tetraacetic acid; EU, endotoxin unit; GOT, glutamic oxaloacetic transaminase; GPT, glutamate-pyruvate transaminase; HBOC, hemoglobin-based oxygen carrier; Hct, arterial hematocrit; HNE, 4-hydroxynonenale; hs-cTnT, high sensitivity troponin T; IHP, inositol hexaphosphate; IMT, 2-imino thiolane; LAL, Limulus amebocyte lysate; LDH, lactate dehydrogenase; LHP, lipid hydroperoxyde; LPS, lipopolysaccharide; MAL-PEG, maleimido polyethylene glycol; PBS, phosphate buffered saline; PEG, polyethylene glycol; TCA, trichloroacetic acid

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https://doi.org/10.1016/j.freeradbiomed.2018.06.018
Received 3 April 2018; Received in revised form 11 June 2018; Accepted 15 June 2018
Available online 18 June 2018

conditions led to a low-affinity HBOC, named Euro-PEG-Hb [9], which, in contrast to MP4, maintained cooperativity in oxygen binding. It did not produce profound hypertension in a rat model and was 99% retained in the plasma within 6 h [9]. Despite the availability of Hb PEG-derivatives endowed with widely different oxygen binding properties, it is still debated whether an oxygen affinity close to that of RBCs is always desirable. Particularly, the extremely low oxygen tensions that can be reached in ischemic tissues might call for high-affinity HBOCs to optimize oxygen delivery where it is most needed [35].

There is evidence that most, if not all, currently available HBOCs tend to produce, besides vasoconstriction and hypertension [36], oxidative-stress, particularly at the level of the myocardium [37,38] and of the kidneys [39]. These effects in humans were shown to be poorly predicted by mouse and rat models, mainly because of a different armory of plasma reducing agents, hampering pre-clinical investigation and leading to disappointing clinical trials [40]. It was suggested that *Cavia porcellus* (Guinea pig), which, as humans and unlike rats and mice, lacks of endogenous ascorbic acid, could lead to a better prediction of the effects of HBOCs in humans [41,42]. Consistently, Guinea pigs or knockout mice not capable of ascorbate synthesis were proposed for the testing of HBOCs [41].

Previous comparisons among HBOCs with different oxygen binding p50 led to the suggestion that hemodynamic effects are related to oxygen binding properties [35]. However, these studies used chemically distinct HBOCs and did not assess in vivo toxicity. In order to understand whether different oxygen binding properties of otherwise similar products affect the oxidative stress and to identify markers of oxidative stress that could be detected days after transfusion, we prepared a high- and a low-oxygen affinity PEGylated Hb with the same modification chemistry and we tested them in a Guinea pig model of transfusion. PEG-Hb^{oxy}, a MP4 [5,22] mimic, and PEG-Hb^{deoxy}, an anaerobically-PEGylated Hb, have already been compared from a biochemical point of view [28]. Oxidative stress markers were assessed in plasma and in protein extracts from heart and kidney.

2. Materials and methods

2.1. Materials

Chemicals were of the best commercial quality available. MAL-PEG was purchased from Iris Biotech GmbH (Marktredwitz, Germany). Immunochemical assays were purchased from Merck Millipore (Darmstadt, Germany), Cell Biolabs (San Diego, CA, USA) or Cayman Chemical (Ann Arbor, MI, USA), as specified for each marker. All other products were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

2.2. Human hemoglobin purification

An aliquot of 45 mL of human whole blood was added to two volumes of endotoxin-free water for red blood cells (RBCs) lysis. The RBC ghosts were separated by addition of solid NaCl (5% w/v) followed by centrifugation at 38000 rpm for 1 h. The resulting Hb solution was filter-sterilized (0.2 μm), dialyzed extensively against the appropriate buffer (depending on the conjugation protocol, *vide infra*) and concentrated to 2.5 mM using a 50 kDa cutoff tangential flow membrane (Repligen, Waltham, MA, USA). All the operations were carried out in sterilized glassware and centrifuge tubes previously washed with caustic soda and rinsed with endotoxin-free water. All reagents stock solutions and buffers were prepared by dissolving the powders in sterile water for parental use. Hb concentration at all steps was assessed by UV–visible absorption using a Cary 4000 UV–VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

2.3. HBOCs preparation

For the preparation of both PEG-Hb^{deoxy} and PEG-Hb^{oxy}, Hb conjugation with PEG was carried out in a two-step procedure that involved: i) thiolation with iminothiolane (IMT) and ii) PEGylation with maleimido-functionalized PEG (MAL-PEG).

For PEG-Hb^{deoxy}, PEG conjugation was carried out under anaerobic conditions in the presence of inositol hexaphosphate (IHP), as already described [28]. The reaction was carried out in a buffered solution containing 50 mM sodium phosphate, 100 mM KCl, 0.5 mM EDTA, pH 7.0, in a 1 L bottle fluxed with filter-sterilized nitrogen, at 20 °C, to which deoxygenated solutions of reagents were added anaerobically. Before PEG conjugation, IHP (1.2 IHP/tetramer ratio) was added to the Hb sample (0.6 mM on tetramer basis). Hb was then reacted with IMT (80 mol/tetramer mol) and, after 1.5 min, with 5 kDa MAL-PEG (12 mol/tetramer mol). The addition of a lysine-containing solution after 25 min and a cysteine-containing solution after 30 min blocked IMT and MAL-PEG reactions, respectively. The sample was dialyzed against phosphate buffer saline (PBS) and concentrated by using a 50 kDa cutoff tangential flow membrane, under aerobic conditions, in order to eliminate unreacted MAL-PEG and other reagents. Finally, the protein sample was passed through a 0.2 μm syringe filter and spun through Proteus NoEndo spin columns (Charles River Laboratories, Wilmington, MA, USA) for endotoxin removal. After the conjugation reaction, PEG-Hb^{deoxy} at 1.64 mM concentration was obtained and stored at −80 °C.

For PEG-Hb^{oxy}, PEG conjugation was carried out aerobically, in the same reaction conditions reported for MP4 (Hemospan, Sangart, USA) [5]. The reaction was carried out in a 1 L bottle at 4–5 °C. Hb (3 mM on heme basis) was treated with IMT (10 mol/tetramer mol) in PBS buffer, pH 7.4 for 4 h before the addition of MAL-PEG (20 mol/tetramer mol). After 2 h of incubation under the same conditions, the PEGylation reaction was terminated by the addition of a cysteine-containing solution (6 mol/MAL-PEG mol). The sample was then dialyzed against PBS and concentrated under aerobic conditions by using a 50 kDa cutoff tangential flow membrane in order to eliminate unreacted MAL-PEG and other reagents. Finally, the protein sample was passed through a 0.2 μm syringe filter and spun through Proteus NoEndo spin columns (Charles River Laboratories, Wilmington, MA, USA) for endotoxin removal. After the conjugation reaction, PEG-Hb^{oxy} at 1.69 mM concentration was obtained and stored at −80 °C.

2.4. Characterization of PEG-Hb^{oxy} and PEG-Hb^{deoxy} oxygen binding properties and purity

Oxygen binding properties and met-Hb content were assessed as already described [3]. Purity, degree of PEGylation and distribution of PEGylated monomers were assessed by image analysis of SDS-PAGE gels, as already described [28].

2.5. Quantification of endotoxins

The content of endotoxins in the final products was assessed using a kinetic LAL test (Endochrome-K, Charles River Laboratories, Wilmington, MA, USA). Five different standard dilutions (50–0.005 EU/mL) were used to produce a log/log correlation of the onset time of each standard with its corresponding endotoxin concentration. The standard curve parameters were then used to calculate the endotoxin levels of the PEG-Hb^{deoxy} and PEG-Hb^{oxy} samples. The positive product control (spiked samples) consisted of PEG-Hb^{deoxy} and PEG-Hb^{oxy} solutions in which endotoxins were added at concentrations around the midpoint of the standard curve.

2.6. Animals preparation

Procedures involving animals and their care were performed in

Table 1
Oxygen binding properties of PEG-Hb^{deoxy} and PEG-Hb^{oxy}. p50 and Hill coefficient (± SE) of HbA, PEG-Hb^{deoxy} and PEG-Hb^{oxy}, in the absence and presence of 5% CO_2 and 200 mM Cl^-, in a solution containing 100 mM Hepes, 1 mM EDTA, at 15 °C, pH 7.0. The Hb concentration was 110–200 μM.

Sample	Stripped		+ CO_2		+ Cl^-		Endotoxin levels (EU/mL)	PEG/ tetramer
	p50 (torr)	n	p50 (torr)	n	p50 (torr)	n		
HbA	2.61 ± 0.05	2.36 ± 0.06	4.09 ± 0.02	2.70 ± 0.04	5.15 ± 0.01	3.08 ± 0.02		
PEG-Hb^{deoxy}[a]	3.18 ± 0.08	2.54 ± 0.19	3.71 ± 0.03	2.21 ± 0.03	3.66 ± 0.05	2.23 ± 0.06	21	5.7
PEG-Hb^{oxy}	0.85 ± 0.02	1.34 ± 0.04	1.23 ± 0.03	1.33 ± 0.06	1.38 ± 0.04	1.44 ± 0.06	14	6.6

[a] Depending on sample concentration, the formation of a dimeric oxygen binding species was observed, with an apparent tetramer-dimer equilibrium constant of 21.5 ± 2.3 μM [28].

Table 2
Hemodynamic parameters and survival of transfused animals. Data are shown as mean ± SD. BL, baseline; PT, post transfusion. Statistical analysis: (A) One-way ANOVA followed by Tukey's multiple comparisons test, comparison between groups at the same time, * ctrl vs PEG-Hb^{deoxy}, # ctrl vs PEG-Hb^{oxy}. (B) Two-way ANOVA followed by Sidak's multiple comparisons test, comparison between groups at the same time, * ctrl vs PEG-Hb^{deoxy}, # ctrl vs PEG-Hb^{oxy}.

	AT	PEG-Hb^{oxy}	PEG-Hb^{deoxy}	P value summary	Statistical analysis
Body weight (g)	401 ± 59	396 ± 86	411 ± 92	ns	
Survival (n/n)	5/7	3/8	6/9	ns	
Heart rate (beats/min)					(A)
BL	213 ± 40	209 ± 24	232 ± 29	**ns**	
Start Transfusion	216 ± 35	212 ± 22	245 ± 30	**	
During Transfusion	190 ± 18	210 ± 6	227 ± 22	**/#	
End Transfusion	188 ± 16	221 ± 11	236 ± 29	#	
PT 30 min	197 ± 18	222 ± 17	218 ± 16	##	
PT 60 min	190 ± 13	219 ± 15	206 ± 20	#	
PT 120 min	197 ± 21	224 ± 16	197 ± 17	**ns**	
Systolic Arterial Pressure (mmHg)					(A)
BL	53 ± 9	54 ± 9	55 ± 6	**ns**	
PT 30 min	51 ± 12	58 ± 8	49 ± 10	**ns**	
PT 60 min	53 ± 8	60 ± 5	60 ± 4	**ns**	
PT 120 min	51 ± 12	64 ± 9	56 ± 13	**ns**	
Mean arterial pressure (mmHg)					(A)
BL	46 ± 8	44 ± 7	48 ± 6	**ns**	
PT 30 min	43 ± 12	52 ± 6	40 ± 10	**ns**	
PT 60 min	42 ± 7	52 ± 10	50 ± 5	*/#	
PT 120 min	41 ± 12	55 ± 8	44 ± 11	**ns**	
Diastolic arterial pressure (mmHg)					(A)
BL	38 ± 9	38 ± 7	41 ± 4	**ns**	
PT 30 min	36 ± 11	44 ± 5	33 ± 9	**ns**	
PT 60 min	36 ± 7	42 ± 7	40 ± 4	**ns**	
PT 120 min	35 ± 8	45 ± 8	34 ± 8	**ns**	
pH					(B)
BL	7.40 ± 0.03	7.5 ± 0.1	7.42 ± 0.03	**ns**	
PT 120 min	7.41 ± 0.02	7.44 ± 0.05	7.42 ± 0.05	**ns**	
pO_2 (mmHg)					(B)
BL	65 ± 14	67 ± 15	78 ± 19	**ns**	
PT 120 min	75 ± 10	78 ± 9	87 ± 10	**ns**	
sO_2 (%)					(B)
BL	91 ± 4	91 ± 4	93 ± 5	**ns**	
PT 120 min	94 ± 3	95 ± 2	97 ± 1	**ns**	
pCO_2 (mmHg)					(B)
BL	50 ± 7	49 ± 9	47 ± 3	**ns**	
PT 120 min	48 ± 4	48 ± 4	45 ± 3	**ns**	
HCO_3					(B)
BL	32 ± 5	32 ± 4	31 ± 3	**ns**	
PT 120 min	30 ± 4	32 ± 4	29 ± 3	**ns**	
Hct (%PCV)					(B)
BL	34 ± 4	38 ± 1	36 ± 2	**ns**	
PT 120 min	34 ± 4	26 ± 4	28 ± 4	**/####	
Hb (g/dL)					(B)
BL	12 ± 1	13 ± 1	12 ± 1	**ns**	
PT 120 min	12 ± 1	9 ± 1	9 ± 1	**/####	

accordance with institutional guidelines in compliance with national and international law and policies. The protocol was approved by the Institutional Animal Care and by the Italian Health Ministry (Legislative Decree no 76/2014- B).

Twenty-four Hartley Guinea pigs (Charles River Laboratories, Italy) weighing 458 ± 134 g were housed in specific pathogen-free conditions with climate control (humidity between 45% and 65%; temperature between 20 °C and 24 °C), with free access to chow and water. After one week of acclimatization, Guinea pigs were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine (Imalgene 1000, Material Italia SPA, Italy) and 5 mg/kg xylazine (Sedaxylan 2%, Happyfarma, Italy); additional doses (Ketamine 30 mg/kg and Xylazine 1.5 mg/kg) were administrated when required to maintain anaesthesia. Under aseptic conditions, a midline incision was

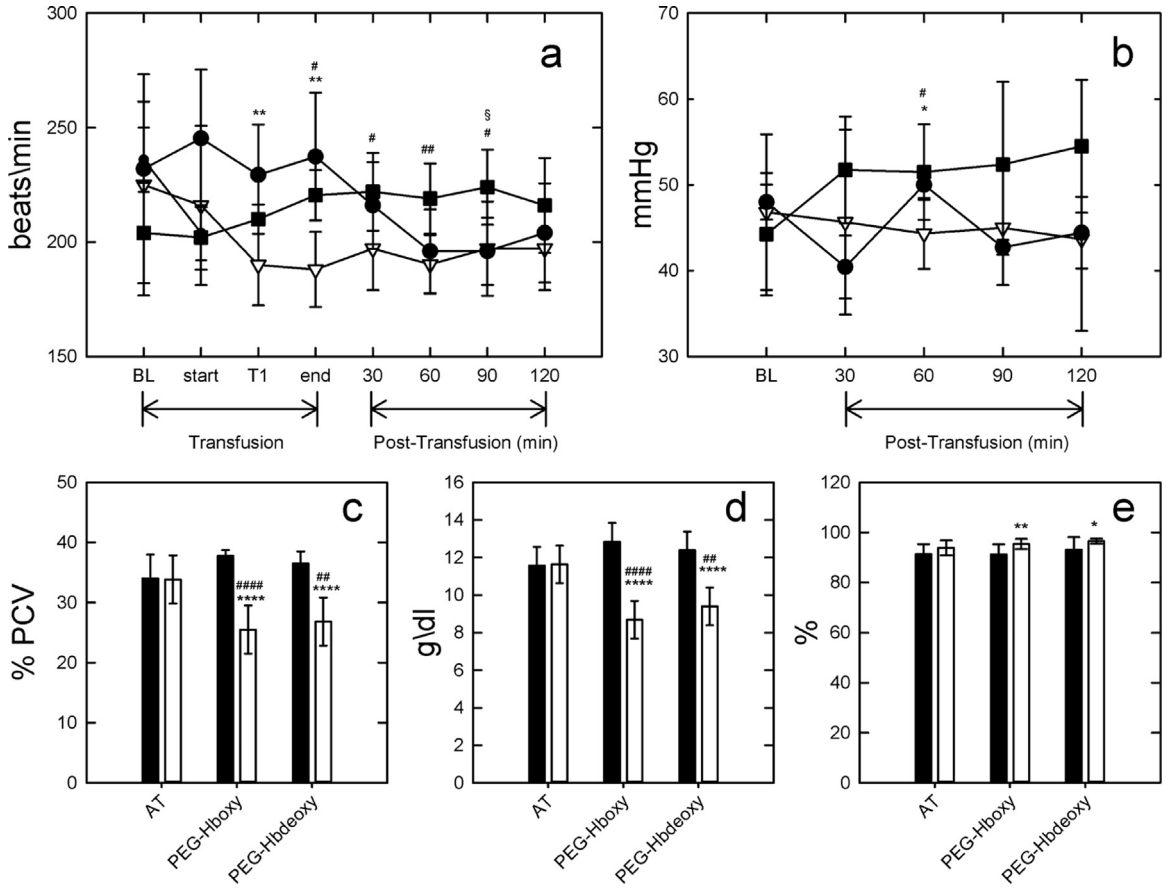

Fig. 1. Hemodynamic parameters upon HBOC transfusion. (a) Heart rates before and after transfusion with PEG-Hb^{deoxy} (closed circles) or PEG-Hb^{oxy} (gray squares) in comparison with the auto-transfusion group (open triangles). Data are shown as mean ± SD. Statistical analysis: Ordinary one-way ANOVA followed by Tukey's multiple comparisons test, comparison among groups at the same time point, * ctrl vs PEG-Hb^{deoxy}, # ctrl vs PEG-Hb^{oxy}, § PEG-Hb^{oxy} vs PEG-Hb^{deoxy}. CTRL n = 7, PEG-Hb^{oxy} n = 8, PEG-Hb^{deoxy} n = 9. (b) Mean Arterial Pressure before and after transfusion with PEG-Hb^{deoxy} (closed circles) or PEG-Hb^{oxy} (gray squares) in comparison with the auto-transfusion group (open triangles). Statistical analysis: Ordinary one-way ANOVA followed by Tukey's multiple comparisons test, comparison among groups at the same time point, * ctrl vs PEG-Hb^{deoxy}, # ctrl vs PEG-Hb^{oxy}, § PEG-Hb^{oxy} vs PEG-Hb^{deoxy}. CTRL n = 7, PEG-Hb^{oxy} n = 8, PEG-Hb^{deoxy} n = 9. (c) Hematocrit, (d) Hb content and (e) oxygen saturation before (black bars) and after 2 h (gray bars) from transfusion. Data are shown as mean ± SD. Statistical analysis: Two-way ANOVA followed by Sidak's multiple comparisons test,* vs own baseline, # vs 2 h ctrl. Ctrl n = 7, PEG-Hb^{oxy} n = 8, PEG-Hb^{deoxy} n = 9.

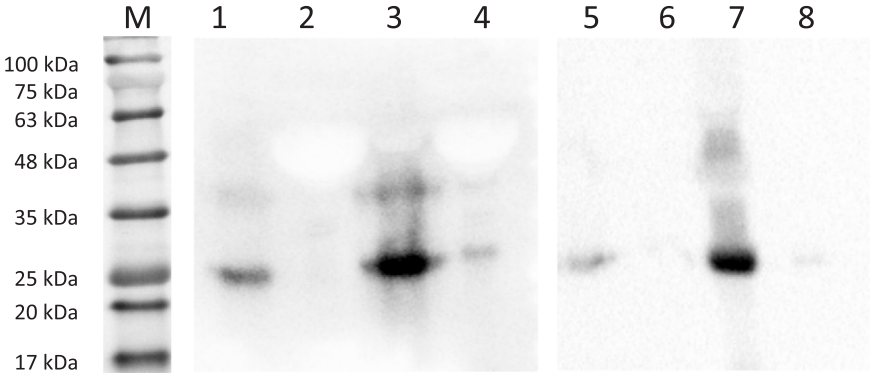

Fig. 2. Western blot of plasma samples pooled from all animals of each group compared with purified HBOCs. **M.** Molecular weight marker, **1.** Purified PEG-Hb^{oxy}, **2.** Plasma pool of animals before transfusion with PEG-Hb^{oxy}, **3.** Plasma pool of animals after 2 h from transfusion of PEG-Hb^{oxy}, **4.** Plasma pool of animals after 7 days from transfusion of PEG-Hb^{oxy}, **5.** Purified PEG-Hb^{deoxy}, **6.** Plasma pool of animals before transfusion of PEG-Hb^{deoxy}, **7.** Plasma pool of animals after 2 h from transfusion of PEG-Hb^{deoxy}, **8.** Plasma pool of animals after 7 days from transfusion of PEG-Hb^{deoxy}.

made around the neck region, allowing for blunt dissection and exposure of the right common carotid artery and the left external jugular vein. A PE-50 catheter was advanced into the right common carotid artery for measurement of the arterial pressure and for blood withdrawal. Another PE-50 catheter was placed in the left external jugular vein for blood or HBOC infusion. All of the catheters were flushed intermittently with saline containing 2.5 IU/mL of heparin. A conventional lead II electrocardiogram (ECG) was continuously monitored. Temperature was measured with the aid of a rectal probe and maintained at 37 ± 0.5 °C throughout the experiment.

2.7. HBOCs administration

Before surgery, animals were randomized to one of the following groups: i) control (auto-transfusion, AT), ii) transfusion with PEG-Hb^{oxy}, iii) transfusion with PEG-Hb^{deoxy}. Fifteen minutes prior to transfusion, baseline physiological parameters were measured. Isovolemic transfusion of 9 mL of whole blood or HBOC was achieved by withdrawal of blood from the carotid artery at 6 mL/h and the concomitant administration of PEG-Hb^{oxy} or PEG-Hb^{deoxy}, maintained at 37 °C, through the jugular vein, at the same rate, using two peristaltic

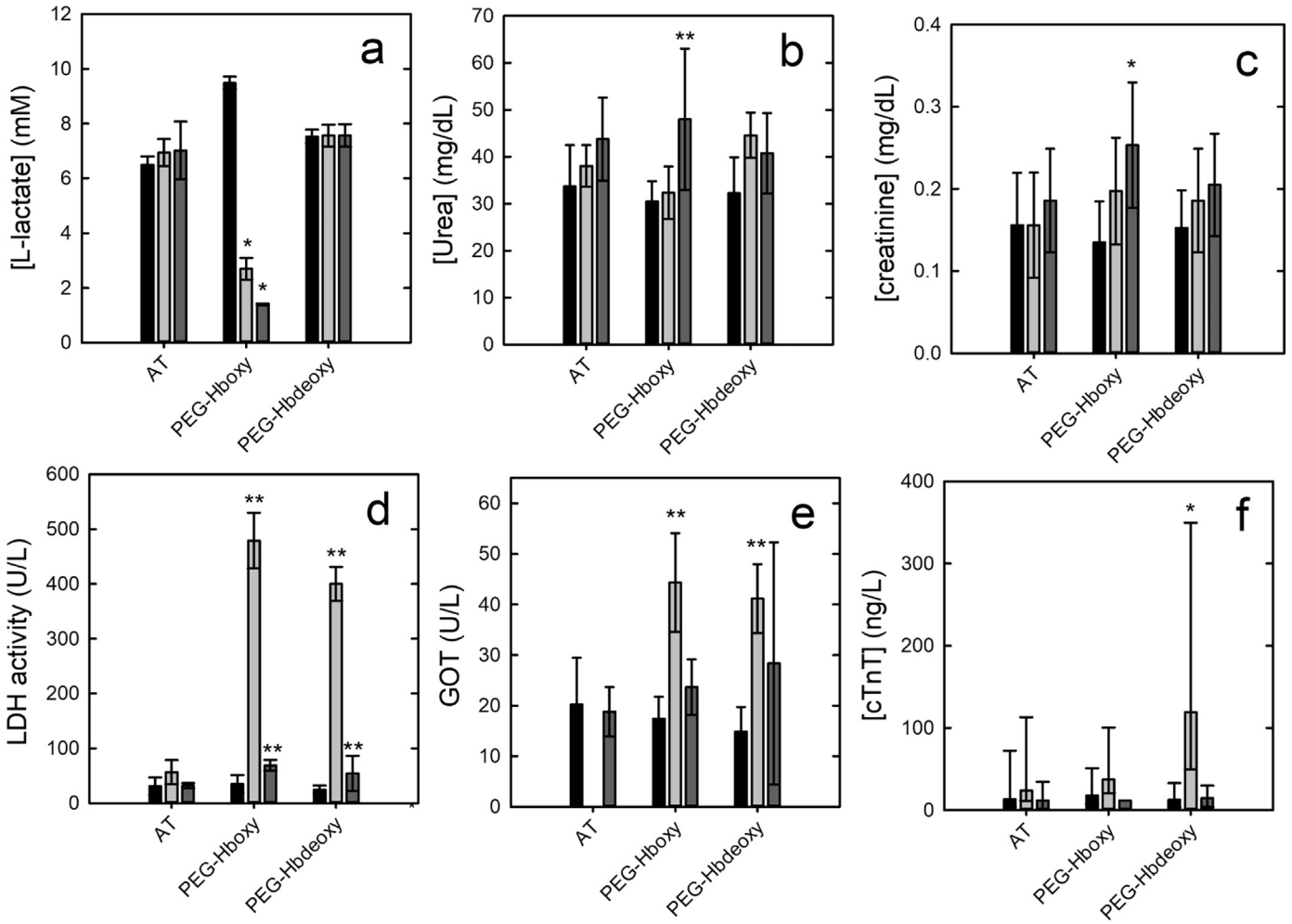

Fig. 3. Selected plasma analytes: (a) L-lactate concentration, (b) urea concentration, (c) creatinine concentration, (d) LDH activity, (e) GOT activity, (f) cTnT concentration for the auto-transfusion group (AT) and for animals transfused with PEG-Hb^{oxy} and PEG-Hb^{deoxy} before treatment (BL, black bars), after 2 h from transfusion (light gray bars) and after 7 days (dark gray bars). Data are shown as mean ± SD (a-e) or median ± IQR (f). Statistical analysis: Ordinary one-way ANOVA followed by Tukey's multiple comparisons test (a-e) or Kruskal-Wallis test followed by Dunn's multiple comparisons test (f). * $p < 0.05$, * * $p < 0.01$ BL vs own PT (at the same time point). AT n = 6/5/5, PEG-Hb^{oxy} n = 8/8/3, PEG-Hb^{deoxy} n = 9/5/5.

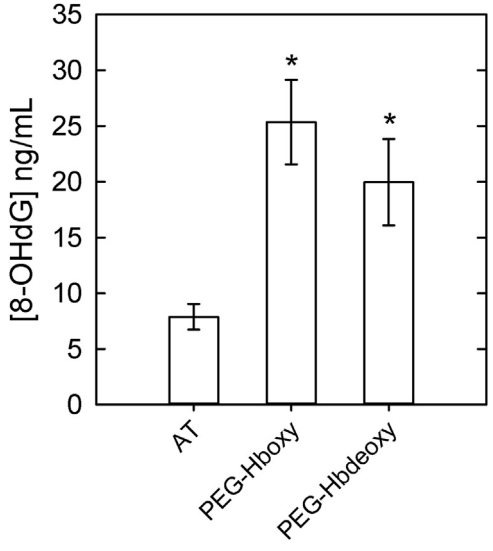

Fig. 4. Plasma levels of 8-OHdG after 7 days from transfusion. Data are shown as mean ± SD. Statistical analysis: Ordinary one-way ANOVA followed by Tukey's multiple comparisons test.* $p < 0.05$ treatment groups vs AT.

pumps (MINIPLUS 3, Gilson, Middleton, WI, USA). In the control group, animals were subjected to auto-transfusion. Following isovolemic transfusion, animals were monitored for 2 h. Plasma was withdrawn for analyses at baseline and 2 h after the transfusion. Catheters were then removed and 0.05 mg/kg buprenorphine (Temgesic, Torrinomedica, Italy), was administered subcutaneously. The animals were then returned to their cages and observed for up to 7 days.

2.8. Blood pressure and blood gas analysis

Aortic pressure was measured with reference to the mid chest with high-sensitivity transducers. Aortic pressure and ECG were continuously recorded on a computer-based data acquisition system supported by CODAS hardware and software (DataQ, Akron, OH, USA). Arterial blood gases and hematocrit were assessed with an i-STAT System (Abbott Laboratories, Princeton, NJ, USA), at baseline and at 2 h after transfusion.

2.9. Blood/tissue collection

After seven days, animals were anesthetized as described above and blood was withdrawn from the abdominal cava vein for biochemical analyses. Blood was collected into EDTA-vacutainers and centrifuged for 2 min at 12,000 rpm. Plasma samples were stored at − 80 °C.

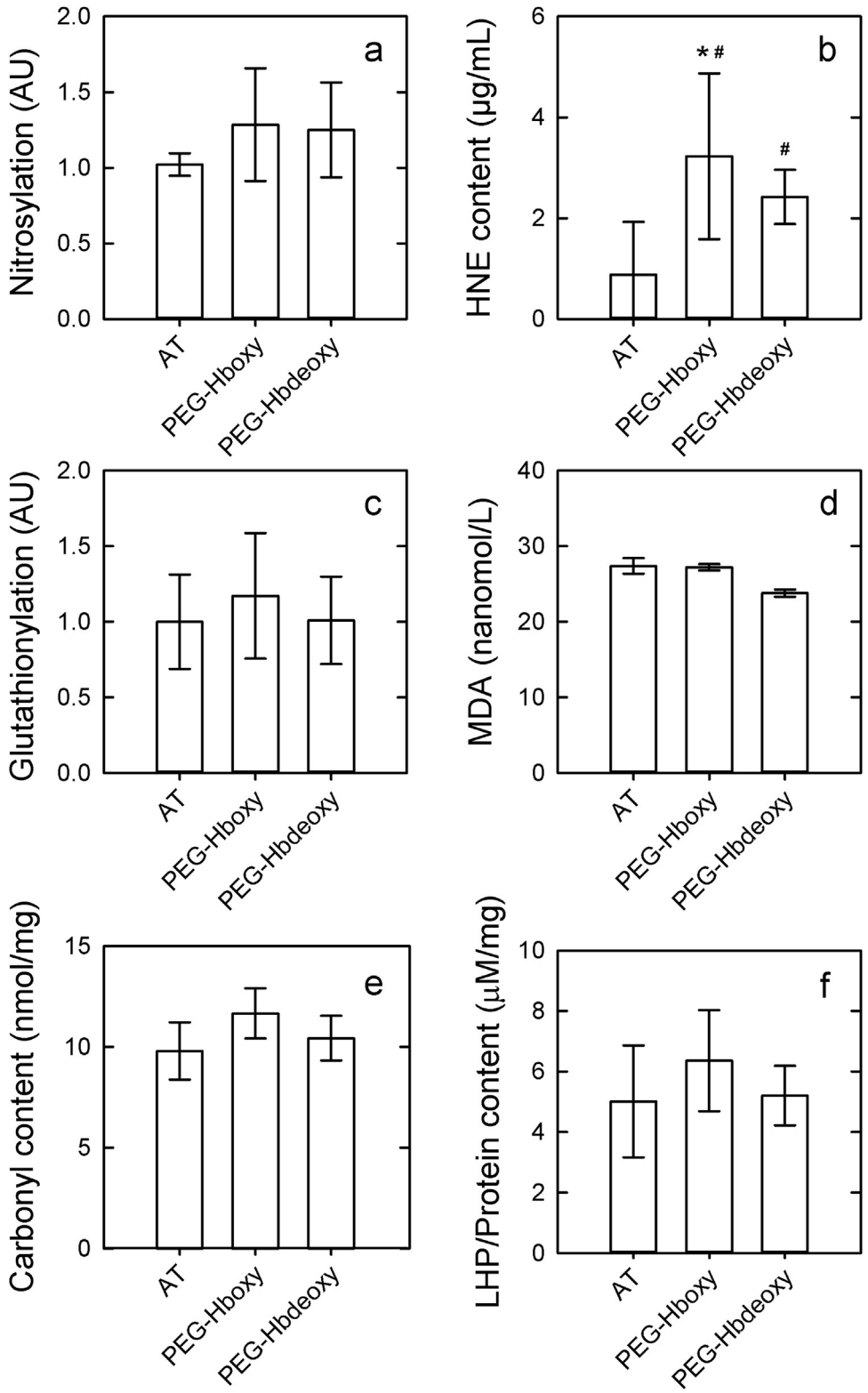

Fig. 5. Markers of oxidative stress in heart extracts. Content of nitrosylated proteins **(a)**, protein HNE-adducts **(b)**, glutathionylated proteins **(c)**, protein MDA-adducts **(d)**, carbonylated proteins **(e)** and lipid hydroperoxides (LHP) **(f)** in heart extracts. Data are shown as mean ± SD. Statistical analysis: Ordinary one-way ANOVA followed by Tukey's multiple comparisons test.* $p < 0.05$ treatment groups vs AT.

Animals were then sacrificed with an intraperitoneal injection of 150 mg/kg pentothal sodium (Pentothal Sodium, Demas, Italy). The heart and kidneys were quickly removed and harvested at − 80 °C for further analysis.

2.10. Determination of residual PEG-Hb^{deoxy} and PEG-Hb^{oxy} in plasma

To determine the residual concentration of HBOCs, aliquots of plasma collected before transfusion, after 2 h and after 7 days were loaded on a 12% SDS-PAGE gel and blotted on a nitrocellulose

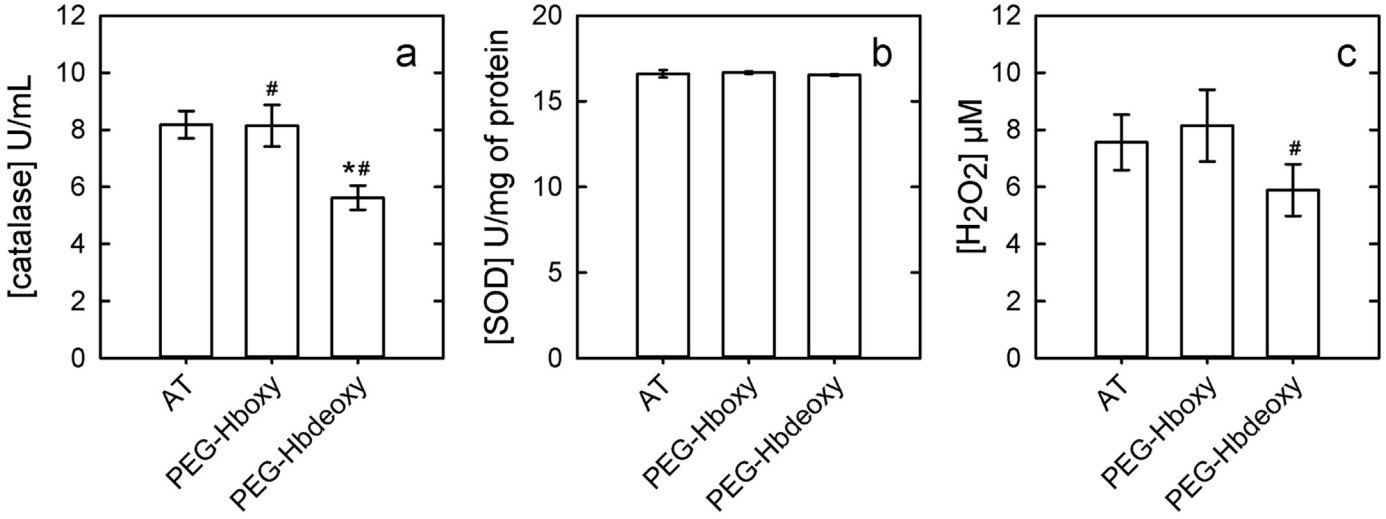

Fig. 6. Antioxidant enzymes in heart extracts. Concentrations of catalase **(a)**, SOD **(b)** and hydrogen peroxide **(c)** in heart extracts. Data are shown as mean ± SD. Statistical analysis: Ordinary one-way ANOVA followed by Tukey's multiple comparisons test.* $p < 0.05$ vs AT; # $p < 0.05$ PEG-Hb^{oxy} vs PEG-Hb^{deoxy}.

membrane, which was then blocked with bovine serum albumin and probed with an anti-Hb antibody conjugated with horseradish peroxidase (HRP)(Abcam, Cambridge, UK). Upon addition of the HRP substrate CheLuminate-HRP PicoDetect (PanReac AppliChem, Darmstadt, Germany), the chemiluminescence signals were collected with a ChemiDoc™ system (Bio-Rad Laboratories, Inc., Hercules, California, U.S.A.).

2.11. Plasma biochemical analysis

All plasma analytes, except for L-lactate, high sensitivity cardiac troponin T (cTnT), 8-hydroxy-2'-deoxyguanosine (8-OHdG) and allantoin were measured with a Roche Cobas INTEGRA 400 analyzer, using commercial reagents provided by Roche Diagnostics (Mannheim, Germany). For lactate dehydrogenase (LDH), PEG-Hb^{oxy} and PEG-Hb^{deoxy} solutions were preliminarily tested for LDH activity to rule out a contribution from human LDH as a contaminant. Briefly, samples of plasma, PEG-Hb^{oxy} solutions and PEG-Hb^{deoxy} solutions at various dilutions in a 10 mM phosphate buffer, pH 7.4 were added of 10 mM NAD^+ (lithium salt) and 10 mM L-lactate. NADH formation was monitored at 340 nm using a Cary 4000 UV–VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). L-lactate levels were measured through its complete oxidation to pyruvate by lactate dehydrogenase in the presence of hydrazine, with equimolar reduction of NAD^+ to NADH, monitored at 340 nm. Plasma hs-cTnT levels were assessed with an electrochemiluminescence assay (ECLIA, Elecsys 2010 analyzer, Roche Diagnostics, Germany). 8-OHdG plasma levels were assessed with an ELISA assay (DNA Damage ELISA Kit - 8-OHdG Quantitation, Cell Biolabs, San Diego, CA, USA) in accordance with the manufacturer's instructions. Allantoin, a biomarker of oxidative stress [43] was measured using a fluorimetric assay based on the enzymatic conversion of S-allantoin to allantoate, followed by acid hydrolysis into glyoxylate in the presence of resorcinol [44].

2.12. Proteins extraction

Individual flash-frozen hearts and kidneys were thawed in ice, extensively rinsed in deionized water, weighed, frozen in liquid nitrogen and cryogenically ground with mortar and pestle. Aliquots of the frozen powders were stored at −80 °C for protein extraction. Powder aliquots of 50 mg were thawed and homogenized by sonication in a buffered solution containing 50 mM phosphate buffer, pH 7.4, 1% DTT and a protease inhibitor cocktail mixture (Sigma-Aldrich, St. Louis, MO, USA). Finally, the homogenate was centrifuged at 15000*g* at 4 °C for 20 min. The supernatant was collected and divided into aliquots for analysis. The total protein content of the extracts was assessed using the Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and was around 1.2 ± 0.3 mg/mL.

2.13. Protein and lipid oxidation of heart and kidney tissue

ELISA assays were carried out in 96-wells plates and the reactions, either chromogenic or fluorogenic, were detected using a plate reader (either a Halo LED 96 - Dynamica Scientific Ltd., Newton Pagnell, UK or a Tecan Spark 10 M, Tecan, Zürich, Switzerland). Electroblotting of SDS-PAGE gels on either nitrocellulose or polyvinylidene fluoride membranes was carried out with a Trans-Blot Turbo Blotting System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For detection, Western blotting membranes were incubated overnight at 4 °C in blocking buffer (2% BSA) and then probed with antibodies or avidin conjugates as specified for each measurement. Absorption spectra were collected with a Cary 4000 UV–VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). All measurements were carried out at least in three independent technical replicates for all the biological samples available. The methods used for the detection of each marker are detailed as Supplementary Materials. For protein carbonylation, four different detection methods were preliminarily tested and compared (Alomari et al., data in brief).

2.14. Image analysis

High-resolution images of the Coomassie-stained SDS-PAGE gel and of Western blots were collected using a ChemiDoc™ system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and analyzed with the Image Lab™ software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The signal of lanes or individual bands of Western blots (expressed as arbitrary units) were divided by the total protein content of the corresponding lane, as quantified by densitometry of the SDS-PAGE gel. For each application, at least three technical replicates were performed.

2.15. Statistical analysis

Statistically significant changes were assessed, as appropriate depending on the data, by one-way analysis of variance (ANOVA), Kruskal-Wallis test or two-way ANOVA, follow by Tukey's Test, Dunn's test or Sidak's test, respectively. In all analyses, a value $p < 0.05$ was

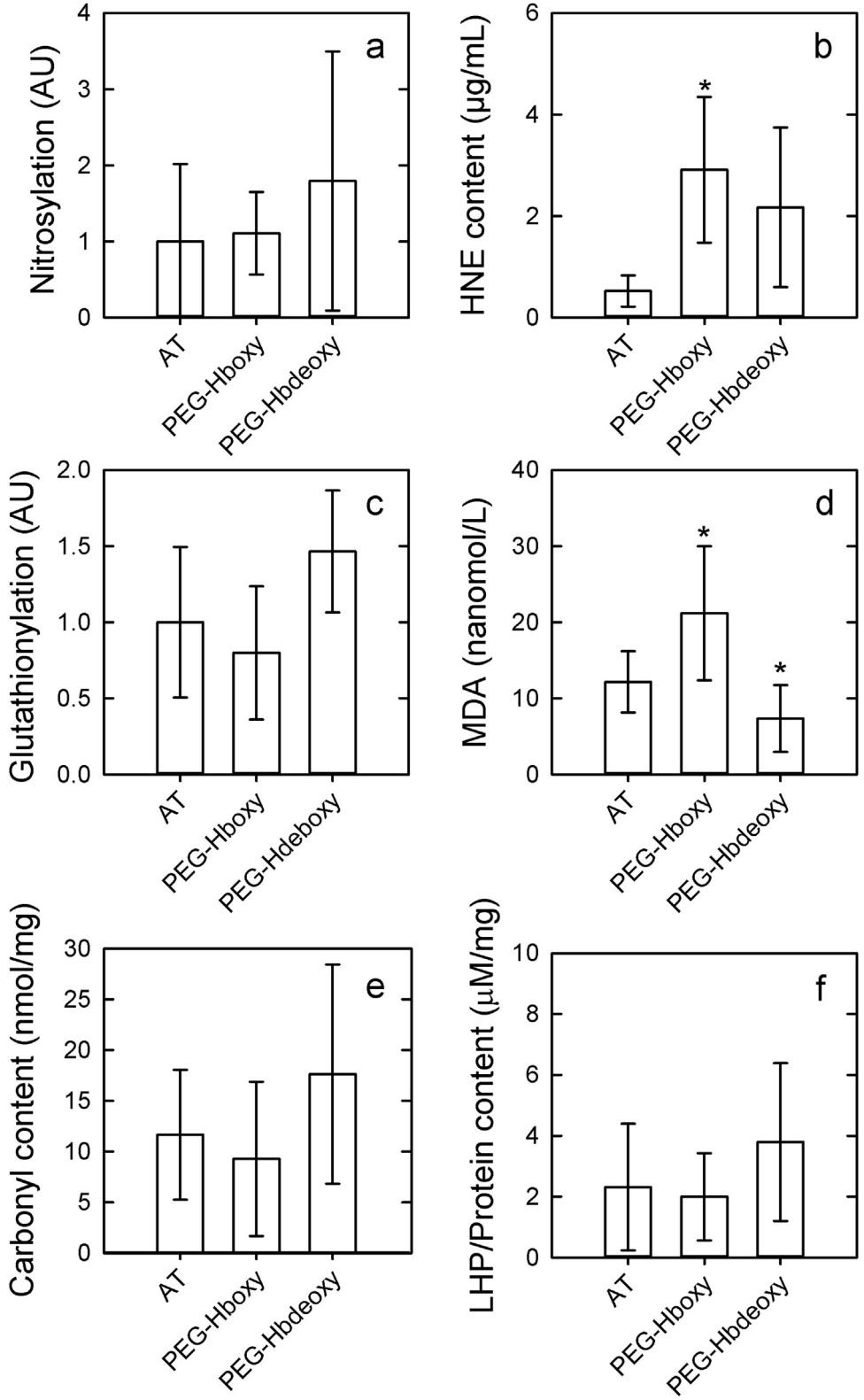

Fig. 7. Markers of oxidative stress in kidney extracts. Content of nitrosylated proteins **(a)**, protein HNE-adducts **(b)**, glutathionylated proteins **(c)**, protein MDA-adducts **(d)**, carbonylated proteins **(e)** and lipid hydroperoxides (LHP) per mg of total protein content **(f)** in kidney extracts. Data are shown as mean ± SD. Statistical analysis: Ordinary one-way ANOVA followed by Tukey's multiple comparisons test.* $p < 0.05$ treatment groups vs AT. ♯ vs other treatment group.

taken as the level of statistical significance. In all plots, values are represented as mean ± standard deviation (SD), or median ± interquartile range (IQR). Parameters resulting from data regression are represented as coefficient ± standard error of the regression (SE). Graphs and statistical analyses were performed using the software SigmaPlot (Systat Software, San Jose, CA, USA).

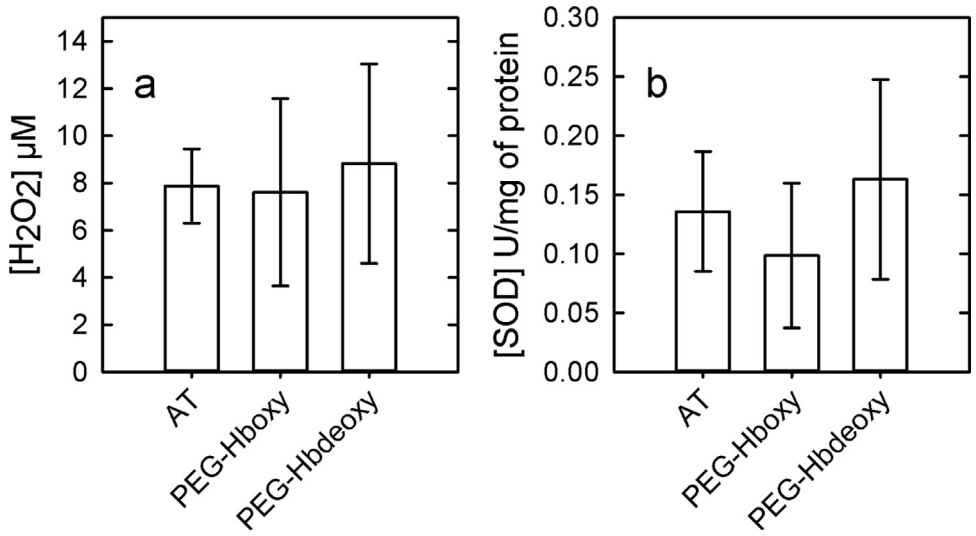

Fig. 8. Antioxidant enzymes in kidney extracts. Concentrations of hydrogen peroxide **(a)** and SOD **(b)** in kidney extracts. Data are shown as mean ± SD. Statistical analysis: Ordinary one-way ANOVA followed by Tukey's multiple comparisons test.* $p < 0.05$ treatment groups vs AT; # $p < 0.05$ PEG-Hb^{oxy} vs PEG-Hb^{deoxy}.

3. Results and discussion

3.1. HBOCs preparation

HbA, PEG-Hb^{deoxy} and PEG-Hb^{oxy} oxygen affinity and cooperativity in the absence and presence of 200 mM chloride ions and 5% carbon dioxide are summarized in Table 1. The PEGylation yield was assessed by densitometric analysis of SDS-PAGE gels, followed by combinatorial calculations, as already described [27,28]. The analysis yielded a PEG/tetramer ratio of 5.7 for PEG-Hb^{deoxy} and 6.6 for PEG-Hb^{oxy} (Table 1). The endotoxin concentration for each preparation is also reported in Table 1. The PEG-Hb^{deoxy} sample contained 21 EU/mL of endotoxin (spike recovery 55%) and PEG-Hb^{oxy} contained 14 EU/mL (spike recovery 50%). The spike recovery values accepted by Pharmacopeia range from 50% to 200%.

3.2. Survival after transfusion

Seven days after blood transfusion, the survival rate was 71.4% (5/7) in the auto-transfusion group, 37.5% (3/8) in the PEG-Hb^{oxy} group and 66.7% (6/9) in the PEG-Hb^{deoxy} group (Table 2). These differences were not statistically significant (Fig. 1S, Supplementary Materials).

3.3. Hemodynamics

Heart rate in the PEG-Hb^{deoxy} and PEG-Hb^{oxy} groups was higher than in the control group during and after transfusion, respectively (Fig. 1a and Table 2). In the PEG-Hb^{deoxy} group, a trend towards a decrease in heart rate, within the physiological range, was observed after the end of the transfusion (Fig. 1a and Table 2). An increase in mean arterial pressure in both treatment groups, compared to the auto-transfusion, was detected 60 min after the end of the transfusion (Fig. 1b and Table 2).

3.4. Blood gas-analysis

Both PEG-Hb^{oxy} and PEG-Hb^{deoxy} groups showed a decrease in Hb and Hct levels 2 h after the end of the transfusion compared to their own baseline and to the control group (Fig. 1c and d). Both results were expected considering that around 26% of the animal blood was substituted with a HBOC solution, based on our calculation developed for isovolemic gradient generation [27]. Despite the loss in RBCs, a moderate increase in sO_2 levels compared to baseline was observed in the treatment groups, indicating that the two HBOCs managed to maintain, and possibly slightly increase, sO_2 levels (Fig. 1e and Table 2). Transfusion with PEG-Hb^{oxy} and PEG-Hb^{deoxy} did not affect pCO_2, HCO_3^- and pH levels (Table 2).

3.5. HBOCs content in plasma

The presence of HBOCs was measured in plasma pools of all animals within each group after 2 h and after 7 days by Western blot, using an HRP-conjugated anti-HbA monoclonal antibody (Fig. 2). The major band at around 25 kDa, corresponding to mono-PEGylated Hb subunits, was evaluated in a semi-quantitative fashion through densitometric analysis (data not shown). After 7 days, plasma PEG-Hb^{oxy} and PEG-Hb^{deoxy} were 13% and 4% of the amount measured after 2 h from transfusion, respectively. The Western blot of plasma samples withdrawn before treatment showed that the anti-Hb monoclonal antibody did not cross-react with any Guinea pig plasma protein nor with its free hemoglobin (Fig. 2).

3.6. Biochemical analysis of plasma

Biochemical analytes were measured in plasma samples withdrawn before transfusion, after 2 h from transfusion and after 7 days. The levels of selected analytes are reported in Fig. 3. The whole panel of analytes is reported in Table 1S (Supplementary Materials).

L-lactate plasma concentration markedly decreased in PEG-Hb^{oxy} animals, both after 2 h and after 7 days from transfusion, whereas it remained unchanged in the control group and in animals treated with PEG-Hb^{deoxy} (Fig. 3a). L-lactate plasma reduction is usually associated to increased oxygen delivery to tissues and reduction of their anaerobic metabolism. This result seems to be correlated with the previous observation that MP4 - a bioequivalent of PEG-Hb^{oxy} - delivers oxygen mostly at the capillary level rather than in the arterioles, unlike the low affinity HBOC PolyBvHb, thus producing higher peripheral oxygenation [35]. Indeed, for the first time and by using two chemically similar products, we correlated the levels of L-lactate, a marker of hyperoxygenation of tissues, with the oxygen affinity of HBOCs. It remains to be established if this metabolic shift toward aerobic metabolism translates into higher oxidative stress.

Biomarkers associated with renal function, including creatinine and urea, showed a general increase after 7 days for all treatment groups, with differences reaching statistical significance only in the group treated with PEG-Hb^{oxy} (Fig. 3b and c). Overall, these results suggested

a diminished renal function, prompting the investigation of oxidative stress markers in this organ (*vide infra*).

Biomarkers of heart function were also investigated. LDH activity, a late marker of heart damage, was measured after ruling out any LDH activity in the HBOCs transfused solutions, which might result from human LDH contamination in the starting material (data not shown). LDH activity exhibited a 10-fold increase after 2 h for both groups treated with HBOCs, but not in the auto-transfusion group, and remained elevated after 7 days (Fig. 3d). LDH activity in the plasma of animals treated with PEG-Hb^{oxy} was significantly higher than that of animals treated with PEG-Hb^{deoxy}, suggesting higher cardiac damage. GOT showed a statistically significant increase after 2 h - within physiological ranges - only in the HBOC-transfused groups (Fig. 3e). GPT did not exhibit any changes (Table 1S, Supplementary materials), suggesting that GOT had myocardial origin. Creatine kinase, also a marker of myocardial damage, slightly increased for all groups after 2 h, but was within normal ranges after 7 days (Table 1S, Supplementary materials).

To further assess a possible heart tissue damage, plasma levels of hs-cTnT were measured and showed increased levels after 2 h in animals transfused with HBOCs, reaching statistical significance only in the group treated with PEG-Hb^{deoxy} (Fig. 3f). After 7 days, cTnT levels were back to normal. cTnT is known to be transiently increased as a consequence of ischemia–reperfusion injury, a tissue damage produced when blood supply returns to the tissue after a period of ischemia but also in non-ischemic conditions [45]. In the latter condition, cTnT levels are usually correlated with tachycardia, in agreement with the observed cardiac parameters (Fig. 1a).

3.7. Oxidation markers in plasma

8-OHdG is a DNA-derived marker of oxidative stress. Its elevated levels in brain preparations of Guinea pig were reported upon transfusion with cell-free hemoglobin to evaluate its effects on the brain blood barrier integrity [42]. 8-OHdG plasma concentration was compared for the three groups of animals after 7 days from transfusion. Plasma levels were three-fold higher in both PEG-Hb^{oxy} and PEG-Hb^{deoxy} groups in comparison with the auto-transfused group (Fig. 4). The differences between the groups treated with PEG-Hb^{oxy} and PEG-Hb^{deoxy} were not statistically significant, although PEG-Hb^{oxy} appeared associated to a slightly higher 8-OHdG plasma concentration.

Allantoin increased after 2 h in all groups, indicating an increased non-enzymatic urate oxidation to allantoin as a response to procedure, but returned to basal levels after 7 days (Table 1S, Supplementary materials). Non-enzymatic conversion of urate into allantoin constitutes an efficient antioxidant system, accounting for 65% of the total free radical scavenging capacity of the human plasma [46]. Urate levels in humans are much higher (around 300 μM) than in Guinea pigs, since urate cannot be enzymatically degraded in hominoids due to the loss by pseudogenization of the enzymatic pathway for oxidative uricolysis [47,48]. Guinea pigs have lost the endogenous capacity of producing the plasmatic antioxidant ascorbate, but maintained a functional uricolytic pathway. Therefore, they lack most of the circulating urate available for the scavenging of oxidant species, suggesting that, at least at plasmatic levels, this animal model is more sensitive than humans in respect to circulating oxidant agents.

3.8. Oxidation markers in heart proteins and lipids

Markers of heart protein oxidation and lipid peroxidation are reported in Fig. 5. Neither S-glutathionylation, S-nitrosylation, carbonylation nor MDA adducts showed any difference between the three groups (Fig. 5c, a, e, d). Also LPH content did not exhibit any statistically significant difference between the groups (Fig. 5f). However, protein adducts with HNE were three-fold higher in both groups of HBOCs-transfused animals in comparison with the auto-transfusion group (Fig. 5b). PEG-Hb^{oxy} appeared to produce a higher elevation in comparison to PEG-Hb^{deoxy}, without reaching statistical significance. HNE adducts were already identified as markers of oxidative stress in the brain of Guinea pigs treated with cell-free hemoglobin [42]. Here, we show that altered levels are detectable in the heart tissue at least for a week after treatment. Among the protein adducts associated to oxidative stress, HNE adducts appeared to be the only relevant markers to assess differences between HBOC and auto-transfusion in the cardiac tissue.

3.9. Catalase, SOD, hydrogen peroxide in heart extracts

Hydrogen peroxide and the antioxidant enzymes catalase and SOD (as total SOD activity) were measured in heart extracts (Fig. 6). Whereas SOD did not exhibit any difference between groups (Fig. 6b), catalase and hydrogen peroxide were significantly lower in the group treated with PEG-Hb^{deoxy} in comparison with the other two (Fig. 6a and c). The decrease in catalase activity might be associated to a ROS-mediated down-regulation of the expression of the enzyme under oxidative stress, as observed in hepatocellular carcinoma [49]. A transient decrease of catalase was also observed in Guinea pigs treated with the polymerized bovine Hb Oxyglobin [39]. It remains to be understood the reason of the significant decrease in catalase upon treatment with PEG-Hb^{deoxy} in comparison with PEG-Hb^{oxy}.

3.10. Oxidation markers in kidney proteins and lipids

Markers of kidney protein oxidation and lipid peroxidation are reported in Fig. 7. Neither S-nitrosylation (Fig. 7a), S-glutathionylation (Fig. 7c), carbonylation adducts (Fig. 7e) or LHP content (Fig. 7f) showed any difference between the three groups. Protein adducts with HNE were significantly higher in both groups of HBOCs-transfused animals in comparison with the auto-transfusion group, with a four-fold difference for PEG-Hb^{oxy}-treated animals (Fig. 7b), the only group exhibiting a statistically significant difference in comparison with the auto-transfusion group. Additionally, unlike in the heart tissue, also the MDA adducts exhibited a significantly higher level for PEG-Hb^{oxy}-treated animals (Fig. 7d), but not for PEG-Hb^{deoxy}-treated animals, possibly indicating a higher kidney toxicity for the former, that contains a much higher concentration of dimers in comparison to PEG-Hb^{deoxy} and is therefore more prone to renal filtration.

3.11. Catalase, SOD and hydrogen peroxide in renal extracts

Hydrogen peroxide and the antioxidant enzymes catalase and SOD (as total SOD activity) were measured in kidney extracts (Fig. 8a and b). Unlike for heart extracts, no significant difference was observed within groups. Catalase activity could not be measured because of the strong interference with the heme content of the kidney extract.

4. Conclusions

An exchange transfusion model in Guinea pig was designed to evaluate the oxidative stress induced by two hemoglobin-based oxygen carriers (HBOCs) modified with the same PEGylation chemistry but endowed with different oxygen binding properties. The concentration of HBOCs was reduced by around 90% in plasma after 7 days from transfusion. L-lactate decrease in PEG-Hb^{oxy} animals confirmed the capacity of high-affinity HBOCs to increase oxygen partial pressure at the capillary level. Increased creatinine and urea levels pointed to a decreased renal function, whereas markers of heart damage – LDH, creatine kinase and troponin – indicated at least transient cardiac toxicity. These results prompted the evaluation of markers of protein, DNA and lipid oxidation in the plasma, in the heart and in kidneys. Both HBOCs produced a significant increase in 4-hydroxynonenal adducts in heart and kidney extracts and of 8-oxo-2'-deoxyguanosine in plasma,

suggesting that these analytes could constitute a platform to evaluate HBOCs-induced oxidative stress. Malondialdehyde adducts increased only in the kidneys of animals treated with PEG-Hb^{oxy} suggesting – in conjunction with a similar trend for creatinine and urea - a higher renal oxidative toxicity in comparison to PEG-Hb^{deoxy}, endowed with lower oxygen affinity. Overall, these results indicate that the oxygen binding properties of HBOCs play a significant role in modulating the degree of oxidative stress.

Funding

We thank the UK Biotechnology and Biological Sciences Research Council for financial support [BB/L004232/1].

Competing interests

C.E.C. and B.J.R. have patents relating to genetic modification of hemoglobin amino acids designed to render a blood substitute less toxic. C.E.C. and B.J.R. are shareholders in a related company (CymBlood).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2018.06.018.

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