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Reversible renal glomerular dysfunction in guinea pigs exposed to glutaraldehyde-polymerized cell-free hemoglobin

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ABSTRACT

Chemically modified hemoglobin (Hb)-based oxygen carriers are promising oxygen replacement therapeutics however their potential renal effects are not fully understood. Using a guinea pig exchange transfusion model, we examined the effects of glutaraldehyde-polymerized bovine hemoglobin (HbG) on the permeability and integrity of the glomerular filtration barrier (GFB), which is comprised of podocytes, fenestrated endothelium, and the glomerular basement membrane. HbG induced marked proteinuria characterized in part by the loss of high molecular weight proteins, including albumin, immunoglobulin, and transferrin, at 4 and 12 h post-infusion that resolved by 72 h. This correlated with HbG-induced GFB alterations based on the reduced expression of specific markers of podocytes (podocin, nephrin, podocalyxin, and Wilms Tumor 1 protein) and endothelial cells (ETS-related gene and claudin-5). Lectin binding studies also demonstrated marked but reversible alterations to the GFB glycocalyx accompanied by increased intraglomerular HbG deposition and 4-HNE protein adduct expression indicative of oxidative damage. Together, these findings indicate that HbG induces reversible glomerular barrier dysfunction in conjunction with transient GFB changes providing new insight into the renal response to chemically modified Hb therapeutics.

1. Introduction

Chemically modified hemoglobin (Hb)-based oxygen carriers (HBOCs) are promising oxygen and volume replacement therapeutics for use in emergency resuscitation, elective surgery, and organ perfusion (Alayash, 2017; Jahr et al., 2012). While red blood cells (RBCs) represent a gold standard of transfusion therapy, HBOCs can address an important need when RBCs are not available, incompatible, or refused based on religious grounds. Currently there are no US-licensed HBOCs for human use, however, a glutaraldehyde-polymerized bovine hemoglobin (Hemopure®) was approved in South Africa for acute anemia settings (Alayash, 2017; Jahr et al., 2012; Mer et al., 2016). Although the continued development and widespread use of these therapeutics has been slowed by lingering safety concerns, their risk-benefit profile may still be favorable in severe anemia situations when RBCs are not an option (Weiskopf et al., 2017). Adverse events reported for some HBOCs in late phase clinical testing include transient hypertension, hemostatic effects, myocardial infarction, and stroke (Silverman and Weiskopf, 2009). Factors believed to adversely influence the safety profile of HBOCs include improper product administration, underlying comorbidities, and the propensity for extracellular Hb to generate and propagate oxidative stress (Biro, 2012; Buehler et al., 2010; Mackenzie et al., 2017; Silverman and Weiskopf, 2009).

Understanding the renal responses to HBOCs remains an important area of investigation given that recipients of these products may be affected by pre-existing conditions including advanced age, diabetes, infection, traumatic shock, and chronic disease that render kidneys more vulnerable to the potential toxic effects of HBOCs (Biro, 2012; Buehler et al., 2010; Herrera et al., 2010; Levien, 2006; Nath et al., 2013; Weinstein and Anderson, 2010). Notably, these disease settings are associated with endothelial dysfunction which represents a potential key risk factor with HBOCs (Biro, 2012; Silverman and Weiskopf,

Abbreviations: Hb, hemoglobin; HbG, glutaraldehyde-polymerized bovine hemoglobin; GFB, glomerular filtration barrier; HBOC, hemoglobin-based oxygen carrier; RBC, red blood cell; GBM, glomerular basement membrane; ROS, reactive oxygen species; WT1, Wilms Tumor 1 protein; 4-HNE, 4-hydroxynoneal; ERG, ETS-related gene; WGA, wheat germ agglutinin; SNA, *Sambucus nigra* lectin; ET, exchange transfusion; TBST, Tris-buffered saline with Tween 20; IgG, immunoglobulin G; BCA, bicinchoninic acid; PBST, phosphate-buffered saline with Tween 20; DAB, diaminobenzidine; DIC, differential interference contrast; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; NT, non-treated sham controls; Lb2, laminin beta 2

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2009; Yu et al., 2010). It has long been recognized that native cell-free Hb resulting from disease- or drug-induced hemolysis is nephrotoxic, particularly when protective plasma protein defenses (e.g. haptoglobin) are overwhelmed (Tracz et al., 2007; Zager and Gamelin, 1989). This has been attributed to the glomerular filtration of dimerized Hb molecules (32 kDa) and, to a lesser extent, native tetrameric Hb that can lead to acute renal tubular necrosis via oxidative and inflammatory mechanisms (Deuel et al., 2016; Tracz et al., 2007; Zager and Gamelin, 1989). Indeed, early HBOC clinical studies employing solutions containing unmodified Hb were beset by renal failure (Savitsky et al., 1978). These early obstacles were partly overcome by chemically modifying Hb using intra- and inter-molecular crosslinking, conjugation, and polymerization techniques to stabilize the Hb tetrameric form and/or increase the overall molecular size to minimize glomerular filtration (Alayash, 2017; Baek et al., 2012; Jahr et al., 2012). Despite these nephroprotective improvements, the potential renal vascular toxicity of HBOCs and/or their breakdown products particularly at the level of the glomerular filtration barrier (GFB) remains poorly defined.

The GFB is made up of three main components: (1) a fenestrated endothelium, (2) a glomerular basement membrane (GBM) consisting of tightly cross-linked type IV collagen and laminin, and (3) podocyte foot processes interconnected by slit diaphragms formed by proteins including nephrin and podocin (Schlöndorff et al., 2017; Scott and Quaggin, 2015). The GFB components are richly decorated with a negatively charged glycocalyx comprised of proteoglycans, glycosaminoglycans and sialoglycoproteins (Schlöndorff et al., 2017). Factors that influence the glomerular passage of proteins from the systemic circulation into the urinary space include their molecular size, electrical charge, and steric configuration. Large (> 40 kDa) and/or negatively charged proteins are less permeable than small and/or electroneutral or positively charged macromolecules. Thus, the presence of large plasma proteins in the urine typically reflects GFB damage (i.e. glomerular proteinuria). Alterations or damage to any of the GFB components can result in proteinuria, and notably, oxidative stress is a major causative factor of GFB dysfunction (Fakhruddin et al., 2017; Haraldsson and Nyström, 2012; Nagata, 2016; Ratliff et al., 2016).

GFB susceptibility to oxidative stress may be particularly important in terms of Hb exposure. Uncontrolled oxidation of cell-free Hb or HBOCs can generate methemoglobin, ferryl heme intermediate, free heme, or iron that can initiate or propagate oxidative damage to cellular lipids, nucleic acids, and proteins (Buehler et al., 2010). Endogenous sources of reactive oxygen species (ROS) can further enhance these oxidative processes and ultimately lead to organ dysfunction or injury when protective mechanisms are diminished or overwhelmed. We previously reported that renal exposure to oxidized human Hb increased oxidative response indicators (nuclear factor erythroid 2-derived-factor 2 and heme oxygenase-1), non-heme iron deposition, lipid peroxidation, and the presence of tubular and glomerular injury markers (Baek et al., 2015). We also reported that exchange transfusion with a glutaraldehyde-polymerized bovine Hb (HbG) increased lipid peroxidation and suppressed the expression and activity of renal antioxidant enzymes at the gene level through epigenetic alterations involving DNA methylation (Rentsendorj et al., 2016). In the present study, we investigate the effects of HbG on the function and integrity of the GFB in a guinea pig exchange transfusion model. The findings herein suggest that the renal exposure to chemically polymerized Hb produces a marked but reversible glomerular dysfunction that correlates temporally with structural GFB alterations.

2. Materials and methods

2.1. Reagents

Oxyglobin (HbG), a FDA-approved HBOC for veterinary use, was purchased from Biopure Corporation (Cambridge, MA). HbG is a chemically modified Hb solution containing a heterogeneous mixture of glutaraldehyde-crosslinked and polymerized bovine Hb at a concentration of 13 g/dl in modified lactated Ringer's. The solution contains unstabilized tetramers (< 5%), stabilized 64 kDa tetramers (~35%), 65–130 kDa oligomers (~50%) and 500 kDa polymers (< 10%). HbG has an average molecular weight of 200 kDa compared to 250 kDa for Hemopure®, a similarly produced glutaraldehyde-polymerized bovine Hb developed for human use. The physicochemical characterization and low immunogenic potential of HbG have been previously described (Buehler et al., 2005; Hamilton et al., 2001). Rabbit polyclonal antibodies to Wilms Tumor 1 protein (WT1), nephrin, Hb alpha chain, albumin, podocalyxin, rabbit monoclonal antibody to podocin, and mouse monoclonal antibodies to laminin beta 2 and 4-hydroxynoneal (4-HNE, clone HNEJ-2) were obtained from Abcam (Cambridge, MA). Mouse monoclonal antibody to ETS-related gene (ERG clone 9FY) was obtained from Biocare Medical (Pacheco, CA). Mouse monoclonal antibody to claudin-5 and Alexa Fluor-conjugated wheat germ agglutinin from *Triticum vulgaris* (WGA) were purchased from ThermoFisher (Walkersville, MD). Fluorescein-labeled *Sambucus nigra* lectin (SNA) (isolated from elderberry bark) and Chitin hydrolysate (a mixture of N-acetylglucosamine monomers and oligomers) were purchased from Vector Laboratories (Burlingame, CA). *Arthrobacter ureafaciens* sialidase was purchased from MP Biochemicals (Solon, Ohio).

2.2. Animal surgical preparation and experimental protocol

Male Hartley guinea pigs were purchased from Charles Rivers Laboratories (Wilmington, MA, USA) and acclimated for one week upon arrival at the FDA/CBER animal care facility. All animals were fed normal diets during the acclimation period and weighed 375–425 g at the time of the study. Animal study protocol was approved by the FDA/CBER Institutional Animal Care and Use Committee with all experimental procedures performed in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). Surgical preparation and catheter implantation were performed as previously described (Buehler et al., 2007). Twenty-four hours after recovery from surgical catheter implantation, fully conscious and freely moving guinea pigs underwent a 50% exchange transfusion (ET) replacing blood with HbG as previously reported (Buehler et al., 2007). Based on our previous studies with this model, the calculated exposure parameters of dose, maximum plasma concentration (Cmax), and area under the plasma concentration versus time curve (AUC0-∞) of HbG following transfusion were 3272 ± 106 mg/kg, 40.0 ± 2.22 mg/mL and 788.1 ± 90.6 mg mL h^{-1}, respectively (Buehler et al., 2007). These exposure parameters were associated with a circulatory half-life of 15.7 h. Sham control animals underwent the surgical procedure and recovered for 24 h. To harvest the kidneys, sham control and HbG-infused animals were euthanized by intraperitoneal injection of Euthasol®, femoral veins were cut, and cold saline was perfused via the arterial catheter to remove blood. Kidneys were dissected, cut in half, and frozen immediately in liquid nitrogen and stored at −80 °C or fixed in 10% formalin for 24 h. Urine samples were retrieved directly from the bladder at the time of sacrifice.

2.3. Urine analyses by SDS-PAGE and Western blot

Urine samples were analyzed for creatinine (Cat# 80189, Crystal Chem, Elk Grove Village, IL) and then diluted to equivalent creatinine concentrations with PBS. Creatinine-calibrated samples were separated on NuPage Novex 4–12% Bis-Tris gels (ThermoFisher) under denaturing and reducing or non-reducing conditions and transferred to polyvinyl difluoride (PVDF) membranes. The membranes were blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% nonfat dry milk and incubated with primary antibodies against albumin, transferrin, or immunoglobulin (IgG). After multiple TBST