Microvascular Research 107 (2016) 83–90

Contents lists available at ScienceDirect

Microvascular Research

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

Sanguinate's effect on pial arterioles in healthy rats and cerebral oxygen tension after controlled cortical impact

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ARTICLE INFO

Article history:
Received 22 February 2016
Revised 23 May 2016
Accepted 6 June 2016
Available online 7 June 2016

Keywords:
Cerebral oxygenation
Hemoglobin-based oxygen carrier
Intravital microscopy
Microcirculation
Oxygen therapeutic
Phosphorescence quenching method
Polyethylene glycol-conjugated carboxyhemoglobin
Traumatic brain injury

ABSTRACT

Sanguinate, a polyethylene glycol-conjugated carboxyhemoglobin, was investigated for cerebral vasoactivity in healthy male Sprague-Dawley rats (Study 1) and for its ability to increase brain tissue oxygen pressure ($PbtO_2$) after controlled cortical impact (CCI) — traumatic brain injury (TBI) (Study 2). In both studies ketamine-acepromazine anesthetized rats were ventilated with 40% O_2. In Study 1, a cranial window was used to measure the diameters of medium — (50–100 μm) and small-sized (<50 μm) pial arterioles before and after four serial infusions of Sanguinate (8 mL/kg/h, cumulative 16 mL/kg IV), volume-matched Hextend, or normal saline. In Study 2, $PbtO_2$ was measured using a phosphorescence quenching method before TBI, 15 min after TBI (T15) and then every 10 min thereafter for 155 min. At T15, rats received either 8 mL/kg IV Sanguinate (40 mL/kg/h) or no treatment (saline, 4 mL/kg/h). Results showed: 1) in healthy rats, percentage changes in pial arteriole diameter were the same among the groups, 2) in TBI rats, $PbtO_2$ decreased from 36.5 ± 3.9 mm Hg to 19.8 ± 3.0 mm Hg at T15 in both groups after TBI and did not recover in either group for the rest of the study, and 3) MAP increased 16 ± 4 mm Hg and 36 ± 5 mm Hg after Sanguinate in healthy and TBI rats, respectively, while MAP was unchanged in control groups. In conclusion, Sanguinate did not cause vasoconstriction in the cerebral pial arterioles of healthy rats but it also did not acutely increase $PbtO_2$ when administered after TBI. Sanguinate was associated with an increase in MAP in both studies.

1. Introduction

A preventable secondary injury mechanism after traumatic brain injury (TBI) is low partial pressure of oxygen in brain tissue ($PbtO_2$). Early intervention to alleviate decreased $PbtO_2$ following TBI is a critical step as the degree and duration of low $PbtO_2$ is related to neurological outcome (Brenner et al., 2012; Chi et al., 2006; Chowdhury et al., 2014). In animal studies of TBI, increasing the inspired oxygen concentration enhanced aerobic metabolism by increasing brain oxygen pressure (Menzel et al., 1999a; Menzel et al., 1999b). Blocking the effects of the hypoxic metabolite adenosine improved cognitive function (Mullah et al., 2014; Mullah et al., 2013) and reduced neuronal death (Varma et al., 2002). Early patient management with normobaric hyperoxia reduced morbidity and mortality (Tisdall et al., 2008; Tolias et al., 2004) and, in particular, treatment regimens targeted to restore $PbtO_2$ can reduce mortality in patients with severe TBI (Narotam et al., 2009).

Hemoglobin-based oxygen carriers (HBOCs) have been successfully used in ischemic conditions to increase the oxygen carrying capacity of blood (Hare et al., 2004), but their clinical development has been halted due to purported side-effects such as vasoconstriction and increases in blood pressure (Natanson et al., 2008). Proximity of cell-free hemoglobin to endothelial cells (Alayash, 2004), extravasation (Urbaitis et al., 1991), and depletion of vascular endothelium derived nitric oxide (NO) by extracellular-hemoglobin (Hb) (Alayash et al., 2007; Doherty et al., 1998; Olson et al., 2004) are the primary hypotheses for the vasoconstriction and increases in systemic and pulmonary blood pressures observed after HBOC infusion. Over the years, structural and chemical modifications have reduced extravasation of the HBOCs, mitigating some of their vasoconstrictive effects (Acharya et al., 2005). Sanguinate (Prolong Pharmaceuticals, South Plainfield, NJ, USA) is composed of a carbon monoxide (CO)-releasing purified bovine Hb conjugated with

Abbreviations: CCI, Controlled cortical impact; HBOC, hemoglobin based oxygen carrier; $PbtO_2$, partial pressure of oxygen in brain interstitium; PO_2, partial pressure of oxygen; PQM, phosphorescence quenching method; TBI, traumatic brain injury.

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http://dx.doi.org/10.1016/j.mvr.2016.06.001
0026-2862/

5000-molecular weight residues of polyethylene glycol (PEG) on the surface lysine residues (Nho et al., 1992) in phosphate-buffered saline. It is a 4% Hb solution with an osmolarity of 310–360 mOsm, and a P_{50} (PO_2 at 50% oxyhemoglobin saturation) of ~11 mm Hg. CO has anti-inflammatory (Ozaki et al., 2012) and vasodilator effects (Leffler et al., 2011). Conjugation with PEG increases the molecular radius to minimize extravasation, increases the circulating half-life of the product (Conover et al., 1997; Conover et al., 1996; Tsai et al., 2006), and increases plasma viscosity (Wettstein et al., 2003). Plasma viscosity may be important for maintaining shear stress-induced NO production by the endothelium and thus may be another mechanism, in addition to the CO, to promote vasodilation (Tsai et al., 2005). Studies have shown that Sanguinate reduces troponin-I after myocardial infarction in mice (Ananthakrishnan et al., 2013) and reduces cerebral infarct volume in rats (Klaus et al., 2010).

Overall, the literature indicates that a treatment regimen, such as administering an HBOC, that increases brain tissue oxygenation without cerebral vasoconstriction could improve long-term mortality and morbidity following TBI. HBOCs afford the additional advantage of not requiring supplemental oxygen, thereby enhancing their potential utility in pre-hospital austere environments such as are encountered on the battlefield. Sanguinate's physiological safety regarding cerebral vasoconstriction and its efficacy in alleviating the $PbtO_2$ reduction after TBI have not yet been tested. The purpose of this study was to evaluate the effects of the HBOC Sanguinate (1) on cerebral microvasculature and systemic blood pressure, and (2) on brain tissue oxygenation after TBI in rats.

2. Methods

The study protocol was reviewed and approved by the Walter Reed Army Institute of Research/Naval Medical Research Center Institutional Animal Care and Use Committee in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as well as all applicable federal regulations governing the protection of animals in research.

Healthy male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), 350–450 g, were anesthetized with ketamine (72 mg/kg IP), acepromazine (4 mg/kg, IP), and buprenorphine (0.1 mg/kg, SC). Acepromazine and buprenorphine were re-administered at 1 h, and ketamine administration was repeated as needed, assessed by the rat's response to toe pinch. A tracheal tube (PE240 tubing) was placed and rats were mechanically ventilated (MouseVent®, Kent Scientific, Connecticut) with 40% oxygen (in order to prevent hypoxia in prone anesthetized rats, as the pilot animals became hypoxic in room air). Ventilator settings were adjusted to maintain normocapnia (35–45 mm Hg). A femoral arterial catheter (PE50 tubing) was placed to continuously monitor blood pressure, heart rate (HR) and to collect arterial blood samples. A femoral venous catheter was used for infusion of Sanguinate, Hextend, or saline. Body temperature was measured using a rectal temperature probe and the targeted maintenance temperature was 37.0 ± 0.5 ° C. The rat's head was stabilized in a stereotaxic frame (Stoelting Co, Illinois).

2.1. Study 1 healthy rats

Access to pial microcirculation was gained through a rectangular craniotomy (~2 mm × 4 mm) drilled in the right parietal bone. The dura was cut and reflected (Levasseur et al., 1975) and the surface of the brain was superfused with artificial cerebrospinal fluid (Harvard Apparatus, MA, USA). A glass cover slip was used to cover the craniotomy to prevent drying of the brain surface. A stereomicroscope (SZ16, Olympus, Japan) equipped with a DP-73 digital camera was used for direct visualization and imaging of pial microvessels. Pial arteriolar diameters were measured at the same locus throughout the experiment using the computer program CellSens (Olympus, 2010). Similar to previous cerebrovascular studies (Abutarboush et al., 2013; Rebel et al., 2006; Rebel et al., 2003), the pial arteriolar vessel diameters were divided into three categories based on size at baseline measurement: (1) “small” vessels having a diameter < 50 μm, (2) “medium” vessels with diameters of 50 to 100 μm, and (3) “large” vessels with diameters > 100 μm. The division into size categories was based on the fact that the amount of smooth muscle increases with vessel size which could lead to differences in degree of vessel contraction (Rosenblum, 1976). All vessel images were acquired at 80× magnification. Prior to euthanasia, a 5% $BaCl_2$ solution was applied topically as a positive control to validate vessel responsiveness since $BaCl_2$ is a known vasoconstrictor in cerebral blood vessels (Rosenblum and Zweifach, 1963).

Rats were randomized to receive Sanguinate (HBOC, n = 10), Hextend control (HEX, n = 11), and no treatment (NON, n = 10) control. For Hextend and Sanguinate, four IV infusions (4 mL/kg per infusion at 8 mL/kg/h rate) were given over 30 min, with 10 min between each infusion, and with a total cumulative dose of 16 mL/kg. Rats in these two groups received 4 mL/kg/h maintenance saline (0.9% NaCl) for the rest of the time while NON rats received 4 mL/kg/h maintenance saline for the whole duration of the study.

Pial arteriolar vessel diameters were recorded at the baseline (Time 0 [T0]) and at the beginning and end of each infusion. Systolic, diastolic and mean arterial pressures, heart rate, and body temperature were recorded at baseline, at the beginning, mid-point, and end of each infusion. Arterial blood samples were obtained at baseline and at the end of each infusion.

2.2. Study 2 TBI rats

A stereotaxic controlled cortical injury device (Leica Impact One®, Leica Biosystems, Illinois, USA) was used to induce a non-penetrating brain injury in the left side of the skull with the center located halfway between bregma and lambda using a 5 mm impactor at 4 m/s velocity, 1 mm depth, and 100 ms contact time. The impactor rod was set at a 16° angle to the vertical plane to maintain a perpendicular position in reference to the tangential plane of the brain curvature at the impact surface. A flexible wire-temperature probe (Physitemp®, Kent Scientific) was placed close to the site of cortical impact to estimate the brain surface temperature and was covered with a skin flap.

For $PbtO_2$ measurements, a rectangular craniotomy (6 mm × 3 mm) was drilled in the right side of the skull between the bregma and lambda, and 1 mm lateral to the midline. A phosphorescence quenching method (PQM) was used to measure brain interstitial oxygen pressure non-invasively. Details of the phosphorescence quenching dependent oxygen measurement equations and technique have been described previously (Wilson et al., 2006). The PQM technique has been used to measure tissue oxygen tension utilizing diffusion of the water soluble molecular probe Oxyphor R2 into spino-trapezius muscle (Song et al., 2013) and brain (Yu et al., 2013). In the current study, a different water soluble molecular probe, Oxyphor G4 (PdG4 molecular probe, Oxygen Enterprises Ltd., Philadelphia, PA) (Esipova et al., 2011), was utilized to measure $PbtO_2$ as described elsewhere (Mullah et al., 2016). In short, Oxyphor G4 was injected in the subarachnoid space and, after a 30-minute incubation period, the outer surface of the dura was washed to remove any leaked oxyphor. To prevent possible atmospheric oxygen contamination through the dura, the craniotomy was covered with a piece of transparent plastic wrap (Cling Wrap, Glad). Two fiber-optic cables (each 2.5 mm in diameter, one carrying excitation light and the other carrying emitted light from the tissue) were positioned on the craniotomy site while the other end of the fiber optic cable was connected to a PMOD5000 fiber-optic phosphorometer (Oxygen Enterprises Ltd.). $PbtO_2$ was continuously monitored at 15-second intervals from baseline (T0) until the end of observation period of 155 min. Values were determined by averaging 8 recordings over a 2 min period.