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Research Report

Perfluorocarbon NVX-108 increased cerebral oxygen tension after traumatic brain injury in rats

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ABSTRACT

Background: Hypoxia is a critical secondary injury mechanism in traumatic brain injury (TBI), and early intervention to alleviate post-TBI hypoxia may be beneficial. NVX-108, a dodecafluoropentane perfluorocarbon, was screened for its ability to increase brain tissue oxygen tension ($PbtO_2$) when administered soon after TBI.

Methods: Ketamine-acepromazine anesthetized rats ventilated with 40% oxygen underwent moderate controlled cortical impact (CCI)-TBI at time 0 (T0). Rats received either no treatment (NON, $n=8$) or 0.5 ml/kg intravenous (IV) NVX-108 (NVX, $n=9$) at T15 (15 min after TBI) and T75.

Results: Baseline cortical $PbtO_2$ was 28 ± 3 mm Hg and CCI-TBI resulted in a $46\pm6\%$ reduction in $PbtO_2$ at T15 ($P<0.001$). Significant differences in time-group interactions ($P=0.013$) were found when comparing either absolute or percentage change of $PbtO_2$ to post-injury (mixed-model ANOVA) suggesting that administration of NVX-108 increased $PbtO_2$ above injury levels while it remained depressed in the NON group. Specifically in the NVX group, $PbtO_2$ increased to a peak 143% of T15 ($P=0.02$) 60 min after completion of NVX-108 injection (T135). Systemic blood pressure was not different between the groups.

Conclusion: NVX-108 caused an increase in $PbtO_2$ following CCI-TBI in rats and should be evaluated further as a possible immediate treatment for TBI.

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Abbreviations: DDFP, Dodecafluoropentane; $PbtO_2$, Brain tissue oxygen tension; PFC, Perfluorocarbons; PQM, Phosphorescence Quenching Method

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1. Introduction

Pre-hospital resuscitation for traumatic brain injury (TBI) patients is an important approach to potentially reduce morbidity and mortality. Hypoxia in the pre-hospital setting may be the single most important secondary injury affecting mortality (Chi et al., 2006; Chowdhury et al., 2014). Tissue hypoxia after TBI can lead to metabolic failure which, in turn, initiates release of excitatory neurotransmitters, adenosine and other inflammatory mediators. In previous animal studies, blocking the receptor of the hypoxic metabolite adenosine attenuated the secondary injury cascade and thus improved behavior outcome in ischemic rats (Melani et al., 2003) improved cognitive function in TBI rats (Mullah et al., 2013) and reduced neuronal death in TBI rats (Varma et al., 2002). In human TBI early intervention to restore tissue oxygen tension is a critical step in reducing secondary brain damage (Chowdhury et al., 2014) as the severity and duration of hypoxia is associated with the outcome (Brenner et al., 2012; Chi et al., 2006). Early patient management with increasing inspired oxygen (normobaric hyperoxia) enhanced aerobic metabolism by increasing brain oxygen pressure (Menzel et al., 1999a, 1999b) and reduced morbidity and mortality (Tisdall et al., 2008; Tolias et al., 2004). In severe TBI patients, mortality was reduced when treatment was adjusted based on brain tissue oxygen tension ($PbtO_2$), and patients who had good outcomes had significantly higher daily $PbtO_2$ (>20 mm Hg) compared to non-survivors who had persistently lower $PbtO_2$ (<10 mm Hg) during the initial two hours of hospitalization (Narotam et al., 2009). Collectively these studies indicate that improved brain oxygenation improves long-term mortality and morbidity following TBI. However, there are currently no unequivocal treatment options available. A pre-hospital therapy that can increase $PbtO_2$ could benefit outcome in TBI casualties.

Perfluorocarbons (PFC) are fluorinated hydrocarbons that are designed to carry oxygen and may be useful to treat hypoxic conditions (Briceno et al., 1999; Riess, 2005). Oxygen dissolving capacity of most PFCs are around 40 ml in 100 ml (Geyer, 1988). PFCs are shown to increase oxygen pressure in tissues in culture discs (Fraker et al., 2013) and in different experimental animal models (see also discussion) (Schroeder et al., 2008). NVX-108 is a dodecafluoropentane (DDFP)-based PFC emulsion (2% w/v DDFP, NuvOx Pharma, Tucson, AZ) which carries over seven times more oxygen *in vitro* at 37 °C compared to perfluorodecalin and perfluoroctylbromide, two other PFCs (Johnson et al., 2009). NVX-108 is a liquid at room temperature but changes to a gaseous state at 37 °C (Correas and Quay, 1996; Johnson et al., 2009). When administered intravenously (IV), intravascular pressure prevents it from forming true bubbles, although the droplets expand and contract (Culp et al., 2012; Johnson et al., 2009). This property appears to enhance the oxygen carrying capacity of NVX-108. Its mean particle size is less than 260 nm (99% of particles remained ≤400 nm at 11 months of shelf life) (Johnson et al., 2009, 2015). The small size of the particles allows them to deliver oxygen to tissue beds where red blood cells (RBC) cannot reach. DDFP improved survival in an anemia model in rats (Lundgren et al., 2006), reduced the myocardial infarct volume in mice (Swyer et al., 2014) and reduced the infarct size in a rabbit stroke model (Culp et al., 2012). The efficacy of this agent to ameliorate brain tissue oxygen pressure reduction after TBI has not yet been investigated.

In this study we applied a non-invasive Phosphorescence Quenching Method (PQM) to measure $PbtO_2$ and tested the hypothesis that NVX-108 would improve $PbtO_2$ when administered soon after moderate TBI in rats.

2. Results

2.1. General observation

There was no difference between the groups in body weight (383±9 g and 434±19 g for NVX and NON, respectively). Rectal body temperature (37.3±0.1 °C and 37.0±0.1 °C respectively) and brain surface temperature (33.7±0.2 °C and 33.7±0.4 °C respectively) were unchanged throughout the study in both groups. The brain surface temperature probe was placed between the brain surface and a flap of the scalp, not within the brain parenchyma, which likely accounted for its measured values being ~3 °C lower than rectal temperature.

In Sham rats, all parameters remained stable throughout the study. Specifically mean arterial pressure (MAP) was 86±3 mm Hg, and heart rate (HR) was 367±34 beats/min.

2.2. Cortical partial pressure of oxygen

In Sham rats baseline (T0) $PbtO_2$ was 25±5 mm Hg and remained stable for the duration of the study period. There were no significant differences in $PbtO_2$ between the NVX and NON groups at baseline (25.1±3.8 *vs.* 31.7±3.2 mm Hg). At 15 min after the injury, $PbtO_2$ in both the groups decreased from baseline by 46±6% ($P<0.001$). Although there were no differences in $PbtO_2$ measurements between groups or across time, the interaction between group and time was significant [F (14, 168)=2.12, $P=0.013$, partial eta squared=0.15]. Specifically, $PbtO_2$ increased over time in the NVX group [F (14, 98)=3.322, $P<0.001$, partial eta squared=0.322], but not in the NON group [F (14, 70)=0.441, $P=0.955$, partial eta squared=0.081]. $PbtO_2$ in the NVX group showed a trend toward increasing from post-injury T15 (14.1±3.2 mm Hg) soon after the first dose of NVX-108, which by T125 became significantly different (18.2±3.7 mm Hg, $P=0.043$). This elevation in $PbtO_2$ above its immediate post-injury level ($P<0.05$) was sustained for the rest of the observation period. The peak increase in $PbtO_2$ occurred 60 min after the second dose of NVX-108 (T135) reaching 19.1±4 mm Hg ($P=0.02$; 143% of T15). In contrast, the immediate post-injury (T15) $PbtO_2$ for the NON group was 16.4±2.0 mm Hg and $PbtO_2$ remained depressed for the rest of the study.

To account for differences between animals across treatment conditions, $PbtO_2$ was also analyzed as a function of percentage (%$PbtO_2$) change from post-injury (T15) (Fig. 1). Similar to the absolute $PbtO_2$ measurements, there was no difference in overall %$PbtO_2$ between groups or across time but the interaction in %$PbtO_2$ change between group and time was significant [F (14, 168)=2.784, $P=0.001$, partial eta

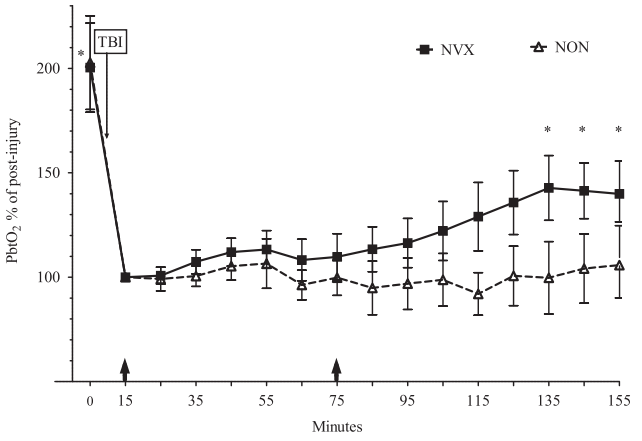

Fig. 1 – Percentage change of $PbtO_2$ from post-injury values (T15) (mean ± SEM). Thin arrow indicates the induction of CCI-TBI following baseline measurements at T0. Thick arrows at 15 and 75 min post-TBI, indicate administration of 0.5 ml/kg IV NVX-108 to NVX rats. *Indicates significant difference ($P<0.05$) within NVX group compared to T15.

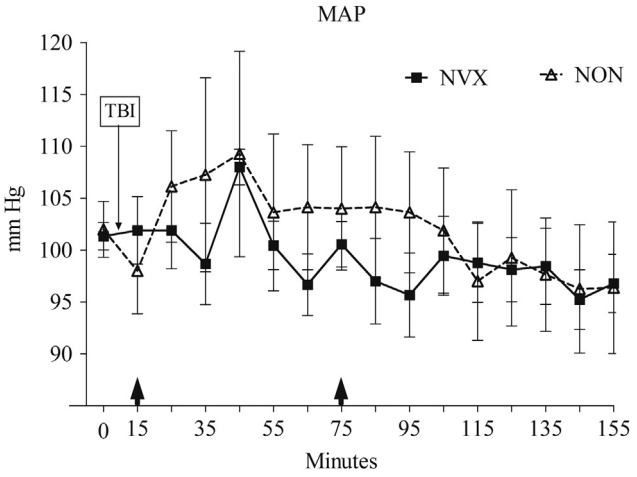

Fig. 2 – Mean arterial blood pressure (MAP; mean ± SEM). Thin arrow indicates the induction of CCI-TBI following baseline measurements at T0. Thick arrows at 15 and 75 min post-TBI, indicate administration of 0.5 ml/kg IV NVX-108 to NVX rats. There were no significant differences in MAP between groups.

squared=0.188]. Similar to absolute values, the post hoc analysis again showed an increase in $\%PbtO_2$ over time in the NVX group [F (14, 98)=4.237, $P<0.001$, partial eta squared=0.377], but not in the NON group [F (14, 70)=0.441, $P=0.918$, partial eta squared=0.081]. Similar to absolute $PbtO_2$ values, the $\%PbtO_2$ in the NVX group was significantly ($P<0.05$) higher from T135 to the end of the study period compared to the immediate post injury values. In the NON group, $\%PbtO_2$ remained at the post injury level for the entire study.

2.3. *Systemic blood pressures and heart rate*

Systemic blood pressures between the NVX and NON groups were not different from each other ($P>0.05$) at any time throughout the study. In both groups, there was a transient increase in MAP following TBI, followed by a gradual decrease over time (Fig. 2).

HR was significantly different between the groups at T0 (416±25 beats/min in the NVX group and 317±22 beats/min in the NON group), making subsequent comparisons between groups invalid. Over time, HR in the NVX group tended to decrease (374±13 beats/min at T155) while HR in the NON group increased slightly (371±34 beats/min at T155). Although the decrease in HR within NVX group is statistically significant over time, HR in both the groups remained within the normal range for anesthetized rats throughout the observation period.

2.4. *Electrolytes and hemoglobin*

Electrolytes (Table 1), pH, glucose and hemoglobin (12.1±0.5 g/dL and 12.8±0.6 g/dL at T15 in NVX and NON groups respectively) were relatively unchanged throughout the study. All values were within normal physiological ranges for this species while ventilated and anesthetized.

2.5. *Histopathology*

Relevant histopathological findings of the brain sections included meningeal congestion, superficial cortical hemorrhage and superficial cortical tissue loss both in (i) the region of the CCI site and (ii) the region of hippocampus. No section received a severity score greater than 3 for any parameter and the modes for all severity categories were similar between groups. Specifically, mode (range) scores for the NON and NVX-108 groups were 0 (0–0) and 0 (0–1) for congestion, 2 (1–3) and 1, 2 (bimodal; 0–3) for hemorrhage and 1 (1–1) and 1 (0–2) for tissue loss, respectively. No statistical analyses of this data were performed.

3. Discussion

This study investigated the effect of the PFC NVX-108 on $PbtO_2$ after moderate CCI-TBI in rats using a PQM technique. Fifteen minutes after CCI-TBI, $PbtO_2$ on the contralateral side of the brain decreased significantly illustrating a global effect of the injury. A cumulative NVX-108 dose of 1 ml/kg (0.02 g/kg PFC) IV was adequate to increase $PbtO_2$ above injury levels.

Other animal studies have shown that PFC administration increases oxygen tension in brain microvasculature in hemorrhaged hamster (Oxycyte, Oxygen Biotherapeutics, Inc., Morrisville, NC or Tenax Therapeutics, Inc., Morrisville, NC) (Cabrales and Carlos Briceno, 2011), in traumatic spinal cord interstitium in rat (Oxycyte, Oxygen Biotherapeutics, Inc.) (Schroeder et al., 2008), and in brain interstitium in a fluid percussion TBI rat model (Oxygent, Alliance Pharmaceutical Corp., San Diego, CA) (Daugherty et al., 2004). However some important differences are to be noted about NVX-108 compared to other PFCs used in those studies. Firstly, in all three mentioned PFC studies $FiO_2=1$ was used, whereas in the current study $FiO_2=0.4$ was used for NVX-108. In this

Table 1 – Plasma Na^+, K^+, HCO_3^- and Lactate (mmol/L, data presented as mean±SEM).

Time	Na^+		K^+		HCO_3^-		Lactate	
	NVX	NON	NVX	NON	NVX	NON	NVX	NON
0	139±1	141±1	4.2±0.2	4.3±0.2	21.5±0.8	21.0±0.8	0.6±0.1	0.4±0.1
15	141±1	141±1	4.0±0.1	4.9±0.2	21.1±0.7	21.0±0.6	0.5±0.1	0.5±0.1
45	139±1	141±1	4.4±0.1	4.7±0.2	21.3±0.6	20.3±0.6	0.5±0.1	0.7±0.1
75	141±1	141±1	4.3±0.2	4.8±0.2	20.0±0.7	20.2±0.7	0.5±0.1	0.7±0.1
105	141±1	142±1	4.4±0.2	4.6±0.2	19.3±0.6	20.0±0.8	0.6±0.1	0.67±0.1
135	141±1	142±2	4.6±0.2	4.9±0.3	19.7±0.8	19.8±0.8	0.5±0.1	0.7±0.1
155	140±1	142±1	4.9±0.2	4.9±0.4	20.2±0.4	19.0±0.9	0.6±0.1	0.7±0.1

study 40% O_2 was used not as supplemental oxygen, but in order to maintain normoxia in prone anesthetized rats, as the pilot animals breathing room air became hypoxic. While it is generally accepted that optimal use of PFCs requires supplementation with 100% O_2 (Kim and Greenburg, 2004; Paxian et al., 2003), previous animal experiments indicated that NVX-108 could be effective in room air (Culp et al., 2012; Woods et al., 2013). There is increasing interest if any oxygen therapeutic can be used with minimal or no supplemental oxygen. The ability to function without need for supplemental oxygen enhances the possibility of use during early prehospital care in austere environments such as the battlefield, where supplemental oxygen may not be readily accessible. Also, not using 100% O_2 may reduce risk of free radical formation or oxygen toxicity (e.g. vasoconstriction). Brain oxygen partial pressure increase with NVX-108 might be even higher if 100% O_2 had been used, although this was not tested. Secondly, the amount of PFCs used in other studies was higher compared to the amount of NVX-108 used in this study. In the spinal cord study 10 ml/kg Oxycyte was injected (Schroeder et al., 2008) and in the fluid percussion TBI model 11.25 ml/kg of Oxygent was injected (Daugherty et al., 2004), and whereas in the current study the cumulative dose of NVX-108 was 1 ml/kg. Thirdly, an increase in MAP was seen in the other PFC studies, ~60 mm Hg in the spinal cord study (Oxycyte) and ~15 mm Hg in the TBI study (Oxygent) (Daugherty et al., 2004; Schroeder et al., 2008). Such an increase in MAP could contribute to any increase in cerebral perfusion pressure and consequently in $PbtO_2$. Our results show that NVX-108 increased the $PbtO_2$ without any increase in MAP.

In the current study NVX-108 had no effect on systemic blood pressures or HR, which is consistent with the findings in a previous study in healthy rats where the same cumulative dose of NVX-108 was not associated with any effect on MAP (Moon-Massat et al., 2014). There were also no adverse effects of NVX-108 treatment on blood gases, hemoglobin, pH, electrolytes, acid–base status, blood glucose, or lactate as all measurements were in the normal physiologic ranges for rats.

NVX-108 was found to increase $PbtO_2$ in CCI-TBI rats. Whether this improvement in brain tissue oxygenation might result in improved clinical outcome from TBI is not clear from this study. No between-group difference in histopathology was detected, but this may be due to the acute nature of the study (2 h and 25 min after CCI-TBI may be too early to detect any cellular changes in standard H&E staining). Considering the very short survival period after TBI, no further advanced staining method was applied for brain histopathology, which may be considered in future survival studies. Neurobehavioral assessment could not be performed as the study was non-survival due to application of several invasive techniques (tracheostomy, craniotomies, multiple vascular lines). Nonetheless, other studies have demonstrated improved outcomes following treatment with a variety of PFCs. In previous animal experiments of acute hemorrhage in rats, PFCs (not NVX-108) were able to preserve organ function (Cabrales and Carlos Briceno, 2011) and improve hepatic microvascular integrity (Paxian et al., 2003). NVX-108 showed improvement in survival and behavior after severe anemia in rats (Lundgren et al., 2006). NVX-108 also improved survival of myocardium after infarction in mice (Swyer et al., 2014) and reduction of the brain infarct size in a rabbit (Culp et al., 2012).

In this study PQM was used to determine interstitial cerebral tissue oxygenation in a TBI model. PQM is a non-invasive method of measuring tissue oxygen tension in the intravascular and interstitial spaces of skeletal muscle and brain (Wilson et al., 2006, 2008; Yu et al., 2013). When the phosphorescent probe is applied to the tissue instead of intravenously, it provides a direct measurement of interstitial (not intravascular) tissue oxygenation. In most instances, the difference between oxygen measurements in a small arteriole/capillary compared to nearby tissue is expected to be small (Torres Filho et al., 1996; Wilson et al., 2006), and arteriolar/capillary oxygen levels are used as proxies for tissue levels. However, for evaluating the efficacy of an oxygen therapeutic fluid, whether or not it is able to appropriately off-load oxygen to hypoxic tissue makes it critical to measure oxygen tension in the tissue interstitium (Weiner, 1994). Thus the decision to measure interstitial tissue oxygen via local probe application was considered essential. Electrode-based methods (e.g., Licox) are commonly used for measuring tissue oxygenation (Daugherty et al., 2004; Mendez et al., 2004; Schroeder et al., 2008). An electrode-based technique provides a combined histogram of intravascular and interstitial oxygen values, and thus the readings have a wide range from intracapillary to intracellular. For this reason these techniques have been criticized as being unable to precisely reflect the oxygen tension within any particular compartment (Weiner, 1994). Nonetheless, the two methods have provided comparable results as our $PbtO_2$ values measured by PQM are similar to a previous rat study using a Licox Clark oxygen electrode (GMS, Kiel, Germany) (Mendez et al., 2004).

Some important study limitations must be noted concerning this experiment. Firstly, the experiment was not a dose–

response study to determine the optimal efficacy or safety of NVX-108 on any of the physiological parameters evaluated. Secondly, general anesthesia may have influenced the results although all groups were treated equally and the types of anesthetics chosen (e.g., ketamine) were selected to have minimal effect on vasoactive function (Ohata et al., 2001). Thirdly, only one superficial region of brain cortex was monitored and it is only an inference that these results are generalizable to the rest of the brain. Earlier studies have reported that the PQM technique records $PbtO_2$ homogeneously across a particular tissue (Tsai et al., 2007) and thus it is possible, although not proven, that our results reflect other regions of the brain. Fourthly, only one type of brain injury was studied and without concurrent injuries to other parts of the body, thus generalizations to clinical scenarios cannot be made with this model. Finally, the smaller size of the rat brain prevented more invasive neurophysiologic and systemic physiological measurements (e.g., intracranial pressures, cardiac outputs or pulmonary artery pressures) while using the PQM technique. Many of the limitations of these studies can be overcome by follow-up studies in more clinically-relevant models of TBI in larger animals (e.g., swine).

4. Conclusion

In summary, CCI-TBI in rats caused a decrease in $PbtO_2$. A cumulative dose of 1 ml/kg NVX-108 successfully increased the $PbtO_2$ when administered soon after TBI. Additional studies are required to determine the optimal dose and timing of treatment as well as to evaluate this product using a more clinically relevant large animal model where additional critical physiological parameters can be measured.

5. Experimental procedures

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 all applicable Federal regulations governing the protection of animals in research.

5.1. *Surgical preparation*

Healthy male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), 350-450 g, were anesthetized with ketamine [72 mg/kg intraperitoneal (IP)], acepromazine (4 mg/kg, IP) and buprenorphine [0.1 mg/kg, subcutaneous (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 inserted and rats were mechanically ventilated with 40% oxygen. Ventilator (MouseVent®, Kent Scientific, Torrington, CT) settings were adjusted to maintain normocapnia ($PaCO_2$=35-45 mm Hg) and 40% oxygen was used to prevent hypoxemia ($PaO_2 \geq 80$ mm Hg) as pilot rats breathing 21% oxygen (room air) had become hypoxemic. Heart rate (HR) was continuously measured using a pulse oximeter (MouseStat® Kent Scientific) with the probe attached to the hind paw. A femoral arterial catheter (PE50 tubing) was inserted to continuously monitor blood pressure and to collect arterial blood samples. A femoral venous catheter was used for infusing maintenance fluid (saline) and treatment (NVX-108). Body temperature was continuously measured using a rectal temperature probe. A target temperature of 37.0±0.5 °C was maintained using a homeothermic blanket (Kent Scientific).

The rat's head was stabilized in a stereotaxic frame (Stoelting Co, Wood Dale, IL). For $PbtO_2$ measurements, a 6 mm × 3 mm rectangular craniotomy was made on the right side of the skull between bregma and lambda, and 1 mm lateral to the sagittal suture. To accommodate the 5 mm diameter impactor tip of the controlled cortical impact (CCI) device, a round craniotomy was made on the left side of the skull with the center halfway between bregma and lambda. A flexible wire-temperature probe (Physitemp®, Kent Scientific) was placed near the brain injury site to monitor the temperature of the brain surface and was covered with a skin flap.

5.2. *Brain tissue oxygen pressure monitoring*

A PQM technique using Oxyphor G4 (PdG4 molecular probe, Oxygen Enterprises Ltd., Philadelphia, PA) was selected to non-invasively measure brain interstitial oxygen pressure with minor modification (Moon-Massat et al., 2015) from a previously described technique.(Esipova et al., 2011; Yu et al., 2013) In brief, Oxyphor G4 was injected into the subarachnoid space and, after a 30 min incubation period, the external surface of the dura mater was washed with body temperature saline to remove any leaked Oxyphor. A few drops of artificial cerebro-spinal fluid (Harvard Apparatus, Holliston, MA; composition: Na 150 mM, K 3.0 mM, Ca 1.4 mM, Mg 0.8 mM, P 1.0 mM, Cl 155 mM;) was applied to the dural surface, and the craniotomy site was covered with a piece of transparent plastic wrap (ClingWrap, Glad) to prevent contamination with atmospheric oxygen. Two fiber-optics (each of 2.5 mm diameter, bundled together) were positioned on the craniotomy site touching the plastic wrap without compressing the brain tissue. The other end of the fiber optics was connected to a PMOD5000 fiber-optic phosphorometer (Oxygen Enterprises Ltd.) for $PbtO_2$ measurement. $PbtO_2$ was continuously monitored at 15 s intervals from baseline [time 0 (T0)] until the end of the observation period of 155 min (T155). A single $PbtO_2$ value was determined by taking an average of 8 measurements over a two minute period.

5.3. *Controlled cortical brain injury*

A stereotaxic CCI device (Leica Impact One®, Leica Biosystems, Richmond, IL) was used to induce a non-penetrating brain injury at T0 using a 5 mm impactor rod at 4 m/s velocity, 1 mm depth and 100 ms contact time as described earlier (Moon-Massat et al., 2015). 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.

5.4. Study design

Animals were randomized to one of the following groups: Sham (no TBI, no treatment, $n=5$), NON (TBI, no treatment, $n=8$), and NVX (TBI, NVX-108, $n=9$). NVX-108 was administered IV (1 ml/kg total dose divided as two 0.5 ml/kg doses) 75 min after the injury. Rats in all groups received maintenance saline (4 ml/kg/h) from the time they were placed into the stereotaxic device until euthanasia. The additional volume of NVX ($\sim$ 0.2 ml per rat) was considered inconsequential and therefore neither Sham nor NON rats received an added volume to match the NVX-108 injection.

$PbtO_2$ and physiologic parameters were recorded at T0 (before injury), 15 min after CCI-TBI (T15) and then every 10 min thereafter. T15 and T75 measurements were recorded just prior to injecting NVX-108. Arterial blood samples were collected before injury, before the injection of NVX-108 and then every 30 min, and were analyzed on an automated blood gas system (ABL 700, Radiometer, Copenhagen, Denmark). At the end of the study, rats were euthanized with 0.5 ml IP Euthasol (Visbac®, Fort Worth, TX) without anesthetic recovery.

Inclusion criteria were set as $PbtO_2 \geq 15$ mm Hg at pre-injury and $\geq 20\%$ reduction in $PbtO_2$ at 15 min post-injury.

5.5. Brain histopathology

The brain of each rat was rapidly collected after euthanasia and fixed in 10% formalin. A matrix-guided trimming protocol for the rat brain was used and one standard brain section was selected from each of the following two regions: (i) the CCI site (which is also closest to the region where $PbtO_2$ was measured) and (ii) the hippocampus region (considered to be the most vulnerable to ischemic injury). Brain sections were embedded in paraffin blocks, cut with a microtome in 5 μm thickness sections, mounted on a glass slide and stained with hematoxylin and eosin (H&E). A board certified veterinary pathologist who was blinded to the treatment groups evaluated the slides for histopathological findings using an Olympus BX-41 light microscope. Superficial cortical tissue loss, superficial cortical hemorrhage and meningeal congestion were graded as follows: 0=normal, 1=minimal, 2=mild, 3=moderate, 4=marked and 5=severe damage.

5.6. Statistical analysis

Data analyses were performed using IBM SPSS Statistics 21.0 (IBM Corporation, Armonk, NY). The data was assessed graphically for normality prior to the inferential statistical tests. Sham rats were used to evaluate the stability of the physiological measurements (i.e., $PbtO_2$ and blood pressures) over time in anesthetized healthy rats and their data were not used in statistical comparisons. NVX and NON groups were analyzed for within-group changes over time as well as being compared to each other. Similar to a previous brain tissue oxygenation study(Moon-Massat et al., 2015) an omnibus 2 (group) $\times$ 15 (observation period) mixed-model ANOVA was used to compare $PbtO_2$, blood pressures, HR, temperatures, blood gases, electrolytes, glucose and lactate data in the NVX group to the NON group. Specifically, this test was used to determine whether the differences between NVX and NON differed as a function of group and observation period. Following this analysis, one-way repeated measure ANOVAs were performed on the experimental groups across the infusion and observation periods. These tests were performed to determine whether the various parameters changed over time. Finally, post hoc comparisons using LSD (least significance difference) were performed to find the effect of drug treatment over time. Independent sample T-tests were used to compare percent change from injury in $PbtO_2$ in NVX and NON at different time points. Due to nearly identical scoring in the histological parameters between the two groups, a formal statistical analysis of the histopathology data was not performed. For all parameters, a P-value ≤ 0.05 was considered statistically significant.

All results are reported as mean $\pm$ SEM.

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Disclosures

The authors declare no conflict of interest for personal or institutional financial interest in drugs, materials, or devices described in their submissions.

Author contributions

S.H.M. performed experiments, analyzed data and wrote the manuscript. B.K.S., R.A. and B.H. performed experiments. A.H. and P.B.W. performed statistical analyses. F.A. performed experiments and analyzed data. P.M. and A.H.S. designed study, analyzed data and wrote the manuscript, C.A. and R.M. M. designed the study and modified the manuscript.

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