



Nitrite pharmacokinetics, safety and efficacy after experimental ventricular fibrillation cardiac arrest

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ABSTRACT

Introduction: Besides therapeutic hypothermia or targeted temperature management no novel therapies have been developed to improve outcomes of patients after cardiac arrest (CA). Recent studies suggest that nitrite reduces neurological damage after asphyxial CA. Nitrite is also implicated as a new mediator of remote post conditioning produced by tourniquet inflation-deflation, which is under active investigation in CA. However, little is known about brain penetration or pharmacokinetics (PK). Therefore, to define the optimal use of this agent, studies on the PK of nitrite in experimental ventricular fibrillation (VF) are needed. We tested the hypothesis that nitrite administered after resuscitation from VF is detectable in cerebrospinal fluid (CSF), brain and other organ tissues, produces no adverse hemodynamic effects, and improves neurologic outcome in rats.

Methods: After return of spontaneous circulation (ROSC) of 5 min untreated VF, adult male Sprague-Dawley rats were given intravenous nitrite (8 μ M, 0.13 mg/kg) or placebo as a 5 min infusion beginning at 5 min after CA. Additionally, sham groups with and without nitrite treatment were also studied. Whole blood nitrite levels were serially measured.

After 15 min, CSF, brain, heart and liver tissue were collected. In a second series, using a randomized and blinded treatment protocol, rats were treated with nitrite or placebo after arrest. Neurological deficit scoring (NDS) was performed daily and eight days after resuscitation, fear conditioning testing (FCT) and brain histology were assessed.

Results: In an initial series of experiments, rats ($n = 21$) were randomized to 4 groups: VF-CPR and nitrite therapy ($n = 6$), VF-CPR and placebo therapy ($n = 5$), sham ($n = 5$), or sham plus nitrite therapy ($n = 5$). Whole blood nitrite levels increased during drug infusion to $57.14 \pm 10.82 \mu$ M at 11 min post-resuscitation time (1 min after dose completion) in the VF nitrite group vs. $0.94 \pm 0.58 \mu$ M in the VF placebo group ($p < 0.001$). There was a significant difference between the treatment and placebo groups in nitrite levels in blood between 7.5 and 15 min after CPR start and between groups with respect to nitrite levels in CSF, brain, heart and liver. In a second series ($n = 25$ including 5 shams), 19 out of 20 animals survived until day 8. However, NDS, FCT and brain histology did not show any statistically significant difference between groups.

Conclusions: Nitrite, administered early after ROSC from VF, was shown to cross the blood brain barrier after a 5 min VF cardiac arrest. We characterized the PK of intravenous nitrite administration after VF and were able to demonstrate nitrite safety in this feasibility study.

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1. Introduction

Cardiac arrest (CA) remains a significant public health burden worldwide with one-half million cases in the US every year [1]. Targeted temperature management confers benefit when applied for 12–24 h after cardiac etiology (primarily ventricular fibrillation [VF]) CA arrest in adults [2–4]. Yet brain injury remains one of the leading cause of death after cardiac etiology CA [5,6] and results in cognitive dysfunction in half of these survivors [7]. Our recent findings [6] confirm earlier studies [8,9] that cardiac etiology CA is phenotypically distinct from asphyxial etiology CA in terms of heart and brain injury which may have important implications in regards to response to drug therapy [6,9]. Thus a persistent need exists for novel neuroprotective therapies to treat CA, and that both pre-clinical and clinical studies should evaluate neuroprotective therapies across CA etiology-based phenotypes.

Nitrite (NO_2^-) is a potential novel therapy for brain and heart ischemia that could improve outcome after CA and/or augment the benefits of hypothermia [10–18]. Mechanistically, nitrite targets a number of important secondary injury pathways that appear to contribute to brain reperfusion injury, such as oxidative stress, mitochondrial injury, bioenergetic failure and secondary hypoperfusion [18–22]. Nitrite is converted locally in ischemic regions to nitric oxide (NO) and participates in cysteine S-nitrosation [18] potentially modulating blood flow, mitochondrial function and free radical generation [12,14,15,17,18,23]. Lower pH results in a significant increase in NO release with heme and molybdenum containing enzymes facilitating nitrite reduction to NO particularly under hypoxic conditions [24–27].

Our prior work demonstrated that a single dose of intravenous nitrite provided during cardiopulmonary resuscitation (CPR) improved cardiac function, survival, and neurological outcomes compared to saline placebo in a mouse-model of potassium chloride-induced CA [16]. In rat models, nitrite dosed shortly after return of spontaneous circulation (ROSC) was shown to reduce neurological damage after asphyxial (pulseless electrical activity) CA [17,18]. It remains to be determined whether nitrite is effective after VF CA which is distinct from asphyxia in having greater cardiogenic shock but less neurological injury [6].

The goal of this study was to answer important questions which remained unresolved after these earlier studies. We determined whether nitrite crosses the blood brain barrier (BBB) and enters the cerebrospinal fluid (CSF) within minutes of dosing after VF similar to our findings in asphyxial CA [18]. We performed detailed pharmacokinetic (PK) modeling never previously reported after CA, which impact dosing and duration (bolus vs. infusion) in ongoing clinical trials (NCT02987088). Our recent phase I study confirmed nitrite safety when dosed during resuscitation after human CA [28,29] nitrite levels in patients were quite variable and only modestly estimated PK models derived in critically ill lung transplant patients [29]. A major objective of this study was to determine how well our human PK models, which were derived in a distinct disease process (lung transplant), recapitulated PK modeling in rat VF CA where ischemic nitrite depletion creates a potential for increased tissue uptake [16]. Finally, we sought to test the hypothesis that nitrite would be neuroprotective when administered immediately after ROSC from VF-induced CA akin to our prior work in asphyxial CA [17].

2. Material and methods

2.1. Rat model

The Institutional Animal Care and Use committee at the University of Pittsburgh, PA, USA, approved this prospective study that included two separate series of experiments. These comply with the ARRIVE guidelines and have been carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals.

Adult male Sprague Dawley rats (400–450 g; Hilltop Lab Animals, Scottsdale, PA) were housed with unrestricted access to food and water. Rats were fasted for 16 h prior to CA. Rats were anesthetized with 4% isoflurane in oxygen, then intubated with a 14-gauge intravenous cannula (Becton Dickinson, Sandy, UT), and placed supine. Rats were mechanically ventilated (Harvard Small Animal Ventilator 683, Harvard Apparatus, Holliston, MA) with a ventilation rate of 45/min. Anesthesia was maintained with 2% isoflurane in FiO_2 0.5 for surgical procedures. Electrocardiogram, respiratory rate, arterial and central venous pressure were continuously monitored and recorded (Powerlab, ADInstruments, CO, USA). Rectal and tympanic probes were used to monitor temperature. Rectal temperature was held at 37.0 ± 0.5 °C with a temperature controlled operating table using lights and a heating pad for heat and a fan for reducing temperature. A Swan-Ganz pacing catheter (size 6F, Edwards Lifesciences LLC, CA, USA) was inserted into the right jugular vein for induction of VF. After surgery, isoflurane was reduced to 1% for 9 min, followed by 0% for 1 min prior to CA to minimize excessive anesthetic effects during the CA period. We obtained two baseline-blood gases and adjusted ventilation as needed prior to the insult. Data on oxygenation, electrolytes and lactate were serially obtained.

2.2. Induction of VF

Immediately before VF induction, mechanical ventilation and temperature control were discontinued. VF was induced over a period of 90 s with 12 V/50 Hz alternating current and was confirmed by a precipitous reduction in the arterial blood pressure and ECG recordings. After 90 s, the voltage was reduced 50% for the remaining 30 s to minimize any electrical injury to the heart. After 2 min of VF induction, the current was turned off, the pacing catheter removed and the vein and skin incision sutured. VF was left untreated for 3 additional min resulting in a 5 min total no flow CA time.

2.3. CPR

Epinephrine 20 $\mu\text{g}/\text{kg}$ and sodium bicarbonate 1 mmol/kg were given intravenously 15 s before start of CPR and flushed by 1 ml of normal saline. CPR was initiated 5 min after VF induction. Mechanical ventilation during CPR was performed at a rate equivalent to that used in the final baseline period (generally 40 breaths/min). Chest compressions were delivered manually with three fingers in the middle of the sternum with equal compression-relaxation periods at a depth adjusted to achieve a systolic blood pressure > 40 mmHg and a rate of 200/min guided by the sound of a metronome (Steinway Metronome, Steinway Musical Instruments, iTunes App). After 60 s of CPR, a single monophasic defibrillation attempt with 20 J was delivered (Physio-Control Lifepak 8 defibrillator, Physio-Control, WA, USA) with immediate resumption of CPR. After 90 s, CPR was briefly paused to assess the blood pressure and ECG. If ROSC had not occurred at that point, CPR was continued and a second dose of epinephrine (20 $\mu\text{g}/\text{kg}$) was given intravenously before delivering a second shock. Defibrillation was repeated every 30 s at 20 J if necessary up to 3 min after initiation of CPR. If ROSC was not achieved, CPR attempts were terminated and the animal excluded from further study.

2.4. Whole blood, CSF and tissue nitrite sampling for PK

Immediately after ROSC, rats were randomized to nitrite or placebo. FiO_2 and ventilation rate were adapted according to arterial blood gas at 5 min post-resuscitation time (RT) based on a standardized protocol. Temperature control was reactivated and rectal temperature was maintained at 37 ± 0.5 °C. Hemodynamics and temperature were measured over 15 min after CPR. Rats received either 1 ml of 8 μM (0.13 mg/kg) sodium nitrite (NaNO_2 in plasmalyte) or vehicle placebo (1 ml plasmalyte) intravenously over a 5-min period from 5 to 10 min

RT in a randomized blinded fashion. Whole blood (50 μ l) was obtained for nitrite measurement at baseline (prior to VF) and after CPR at 5, 7.5, 10, 11, 12, 13, 14 and 15 min RT. After each blood sampling we returned an initial 100 μ l blood waste and flushed our lines with 50–100 μ l plasmalyte. This represents data from the time period during and immediately after the therapeutic infusion. After 15 min, rats were placed into the prone position and CSF was obtained from the cisterna magna using a 25G 5/8 needle.¹⁴ CSF was centrifuged and the supernatant frozen. Rats were transcardially perfused with nitrite-free water and brain-, liver- and heart-tissue were extracted and homogenized in a cyanide-based nitrite preservation solution [16,17]. Whole blood samples for the assessment of nitrite were preserved using a ferricyanide-based solution [16]. CSF, tissue and blood samples were analyzed by a tri-iodide based reductive chemiluminescent method using an ozone chemiluminescence detector (Model 280i nitric oxide analyzer [NOA], Sievers Instruments, CO, USA) to measure nitrite levels [16,17].

2.5. Rat VF survival study

The same CA and resuscitation protocol described above was used for the 8-day survival experiments which assessed PK and outcomes. This was a randomized, investigator-blinded study. After ROSC, rats were randomized to NaNO₂ (8 μ M) vs. placebo therapy delivered in blinded fashion over a time period of 5 min from 5 to 10 min RT. Whole blood nitrite levels were measured at baseline, 4, 11, 18, 25, 30, 45 and 60 min. These data were combined with early nitrite samples obtained in the experiments above to a dose-concentration nitrite PK model. Weaning from the ventilator was performed at 30 min RT, extubation and removal of central catheters were carried out at the end of ICU phase (60 min RT). Using a midline laparotomy incision, a Mini-mitter probe (Mini-Mitter Co., Sunriver, OR) was placed into the peritoneal cavity to allow postoperative temperature control (37 °C for \geq 16 h after ROSC) and continuous monitoring of movement. Rats received a subcutaneous bolus of 10 ml dextrose 5% in 0.9% sodium chloride (D5NS) before being returned to their cage. Rats were allowed unrestricted access to food and water and were monitored for weight and activity every 24 h by a technician.

2.6. Sham control group

Sham rats underwent the same surgical procedures, blood withdrawals and testing as the CA rats, but no CA was induced. Five ml of plasmalyte were administered over 5 min at RT 5 of 60 min. An ICU phase of 60 min was performed during which sham animals remained intubated and sedated.

2.7. Neurologic outcomes

Neurologic Deficit Score (NDS) [30] was assessed at post-arrest days 1, 2 and 8. On day 7, a 2-day-fear conditioning protocol [6,31] was initiated. Rats were placed in a Plexiglas conditioning box (Habitest operant cage, Coulbourn Instruments, Holliston, MA) with steel grid bottom. Rats were conditioned to associate a tone (85 dB; 3 kHz) for 10 s with brief pain delivered by an electrical foot shock (1.0 mA over 1 s). Conditioning consisted of 5 cue-shock cycles each consisting of 60 s of silence and 10 s of cue with a shock in the final sec (total time 420 s). On day 8, rats were again placed in the Plexiglas box with the floor and walls disguised and a distracting odor and soft white noise (60 dB; 500 Hz) intended to conceal the contextual clues that this is the same chamber where shocks were performed the day prior. Rats were allowed to explore 120 s at which point the conditioned tone was applied for 60 s without shock followed by 60 s silence (total 240 s). During this entire period, freezing (remaining motionless) is recorded via video and quantified using FreezeFrame (Coulbourn Instruments, Holliston, MA). Cue mediated freezing was calculated as the percent time spent frozen during the 2 min after the start of the conditioned cue on day 2,

indicative of memory and extinction, minus the percent time frozen in the initial 2 min which is indicative of non-specific sensitization [32].

On day 8, after completion of the fear conditioning testing, rats were anesthetized with 2–4% isoflurane and trans-cardiac perfusion with paraformaldehyde was performed followed by necropsy. Brains were removed, further fixed and sectioned. Formalin-fixed brains were divided into 8 coronal slices 3 mm apart and embedded in paraffin blocks. Five μ M sections were stained with haematoxylin and eosin (Millipore, Temecula, CA). Surviving hippocampal CA1 neurons were then quantified using haematoxylin and eosin stain (H&E) and counted.

2.8. PK analysis

Whole blood nitrite concentration vs. time profiles for each rat were analyzed individually by nonlinear regression using WinNonlin Phoenix (Pharsight, Mountain View, CA). Profiles were simultaneously fit to an intravenous infusion, no lag time, 2 compartmental, first order elimination model based on inspections of goodness of fit. Calculated parameter estimates included predicted maximal concentration (C_{Max}), apparent volume of distribution at steady state (V_{SS}), systemic clearance (CL_s), and terminal elimination half-life (beta half-life, $T_{1/2,\beta}$) and were expressed as mean \pm standard error.

2.9. Statistics

Data are reported as mean and standard deviation or median and interquartile range, if not normally distributed. Statistics were performed with STATA SE 13.0 Statistics (College Station, TX). Group comparisons were made with a one-way analysis of variance and a post-hoc Bonferroni adjustment. Comparisons of 2 groups were performed with *t*-test (parametric) or Mann Whitney *U* test (non-parametric). Figures were designed with GraphPad Prism 7.0 (La Jolla, CA). A two-tailed *p*-value less than 0.05 was considered as statistically significant.

All authors had full access to the data and take full responsibility to the integrity of the data. All authors have read and agreed to the content of the manuscript as written.

3. Results

3.1. Nitrite tissue distribution and pharmacokinetics

In the first series of experiments, rats ($n = 21$) weighing 427 ± 12 g and were randomized to 4 groups after ROSC: VF and nitrite therapy ($n = 6$), VF and placebo therapy ($n = 5$), sham ($n = 5$), or sham and nitrite therapy ($n = 5$). Time to ROSC was not different between VF groups (VF and nitrite 111 ± 60 s, VF and placebo 152 ± 58 s; $p = 0.28$). CSF was withdrawn at 17 ± 1 min after start of CPR with no difference between groups. CSF sampling was successful in 17/21 rats due to the technically challenging nature of the procedure.

Nitrite levels in CSF at 15 min increased significantly compared to placebo whether dosed after VF or sham surgery (Fig. 1A) with the increase exceeding 10-fold greater than placebo. CSF nitrite levels were significantly higher than all tissue levels ($p = 0.02$ to < 0.0001). Brain (Fig. 1B), heart (Fig. 1C) and liver (Fig. 1D) tissue all had more modest 2-3-fold increases in nitrite level compared to placebo which were similar between VF and sham. Liver levels of nitrite were significantly lower in nitrite-treated sham ($p = 0.024$) and VF ($p = 0.01$) rats compared to brain with a similar trend ($p = 0.056$ – 0.09) noted between heart and liver (Supplemental Fig. 1). We did not observe significant nitrite depletion after CA comparing the placebo treated VF group to sham. Due to technical reasons, 3 samples of included animals could not be analyzed. Simultaneous levels of nitrite (μ M) in whole blood, CSF and brain tissue at approximately 15 min RT after VF/treatment were 23.4 ± 2.5 , 10.0 ± 4.0 and 5.6 ± 1.0 .

Whole blood nitrite levels increased during drug infusion to

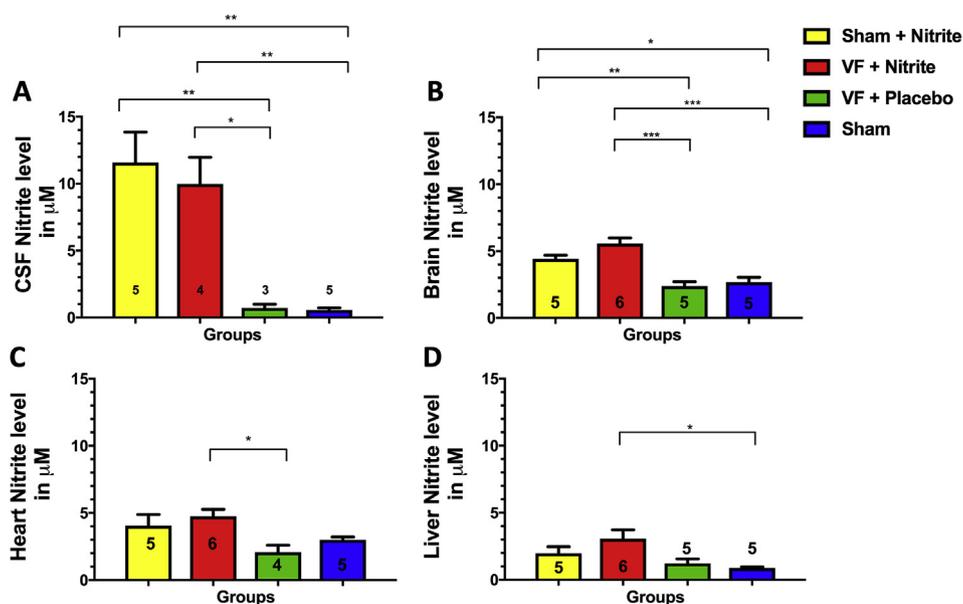


Fig. 1. Results of the tissue 15-min model. Nitrite levels in (A) CSF, (B) brain, (C) heart and (D) liver tissue homogenates. Data are shown as mean (± standard error). Numbers of rats per group are shown within each bar.

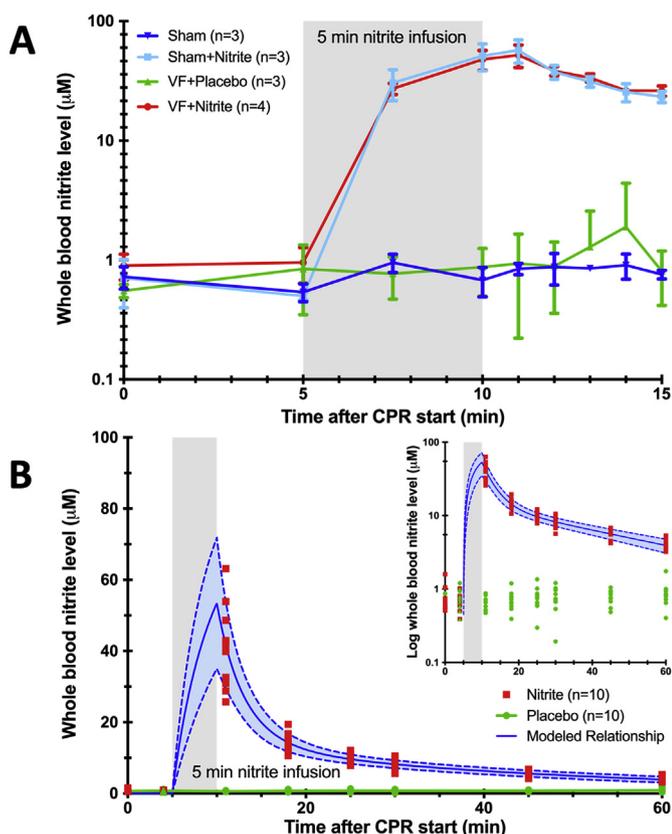


Fig. 2. Pharmacokinetics (PK) of nitrite in blood. (A) Whole blood nitrite levels 15 min after start of resuscitation from short term experiments (CSF and tissue harvest after 15 min). (B) Whole blood nitrite levels up to 60 min after start of resuscitation from 8-days survival experiments including best fit PK modeling (solid line is the mean and dashed line is the standard deviation).

57.14 ± 10.82 µM at 11 min RT (1 min after dose completion) in the VF nitrite group vs. 0.94 ± 0.58 µM in the VF placebo group (p < 0.001) (Fig. 2A). Whole blood nitrite concentrations over time were best fit by a two-compartment model for all rats (Fig. 2B). PK profiles exhibited a rapid distribution phase (declining 65% from peak

values in the first 7 min following the completion of the infusion) with a more gradual terminal elimination (Fig. 2B). In VF rats, the model predicted maximal concentration was 53.43 ± 3.81 µmol/L, the apparent volume of distribution at steady state (V_{SS}) was 0.69 ± 0.13 L/kg, the systemic clearance (CL_S) was 22.52 ± 2.06 mL/min/kg, and terminal elimination half-life (beta half-life, $T_{1/2,\beta}$) was 29.66 ± 8.73 min. VF (compared to sham surgery) had little or no impact on both nitrite PK and endogenous levels as evidenced by these nearly superimposable concentration-time profiles.

3.2. Outcomes from VF in rats treated with nitrite vs. placebo

We randomized 20 rats to nitrite vs. placebo-treatment delivered 5 min after ROSC. Median weight was 431 ± 14 g. Nineteen of 20 (95%) rats randomized survived until day 8. One rat in the placebo group died at 2 days after VF. CPR-duration in the nitrite group was 99 ± 39s vs. 118 ± 31s in the placebo group (p = 0.86). Between groups, no significant differences were found in baseline labs or physiologic variables. (Table 1/Fig. 3). No differences were noted in arterial blood gases at 5 (post-VF but pre-drug therapy) or 15 min (post-drug therapy) after VF (Table 2/Fig. 3). No significant differences were noted in NDS at days 1, 2 or 8 (Fig. 4A), CA1 neuronal survival (Fig. 4B) or freezing after conditioned fear (Fig. 4C) between nitrite and placebo

Table 1
Baseline characteristics prior to cardiac arrest, mean (± standard deviation).

Parameter	Placebo (n = 9)	Nitrite (n = 10)	P-value
pH	7.40 (0.02)	7.41 (0.03)	0.49
BE (mmol/l)	-0.5 (2.2)	-0.1 (2.2)	0.67
Lactate (mmol/l)	0.9 (0.3)	0.9 (0.3)	0.96
pO2 (mmHg)	97 (11.4)	106 (18.7)	0.24
pCO2 (mmHg)	37.9 (4.4)	39.4 (3.6)	0.46
Hb (g/dl)	14.8 (1.5)	14.9 (0.5)	0.96
Na ⁺ (mmol/l)	142 (3)	144 (3)	0.37
K ⁺ (mmol/l)	3.7 (0.6)	3.8 (0.4)	0.63
MAP (mmHg)	83 (8)	82 (9)	0.81
Heart rate (bpm)	339 (12)	321 (27)	0.08
Body Temp. (°C)	37.0 (0.4)	37.1 (0.3)	0.50
Weight (g)	435 (13)	427 (15)	0.24

Abbreviations: BE: base excess; Hb: Haemoglobin; Na⁺: Sodium. K⁺: Potassium; MAP: mean arterial pressure; bpm: beats per minute.

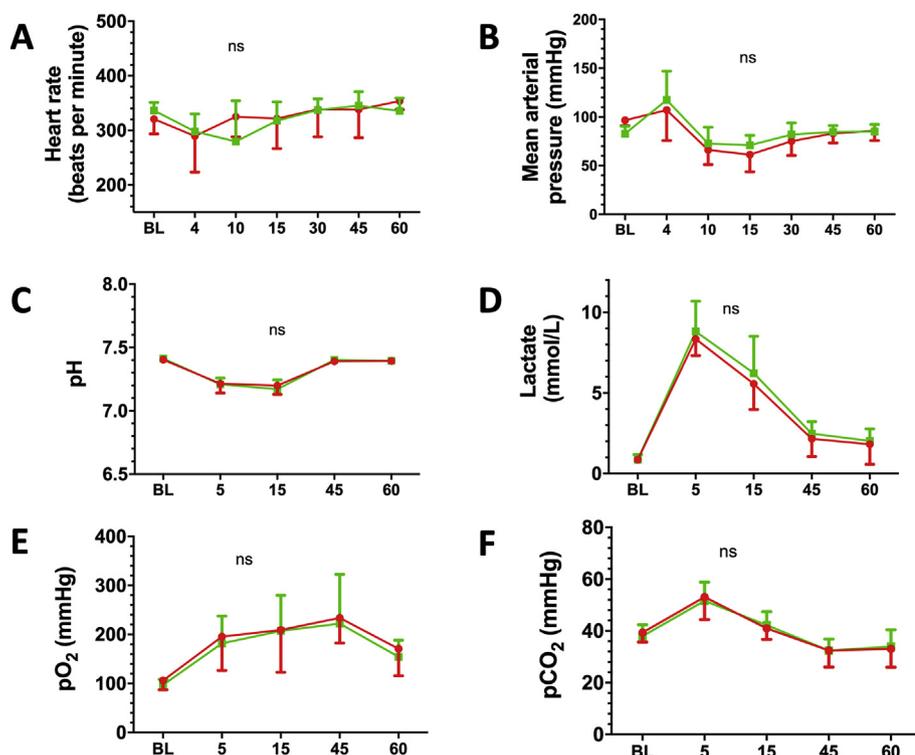


Fig. 3. Physiological and biochemical results of the 8-days survival model. Sequential measurements of physiologic and biochemical variables were made at baseline (BL) and after defined times (in minutes) after return of spontaneous circulation (e.g. 5 = 5 min after ROSC). Over a total time period of 60 min during ICU phase, Nitrite (red circles, $n = 10$) and placebo (green circles, $n = 9$) treated animals showed no significant difference in (A) heart rate, (B) blood pressure, (C) arterial pH, (D) lactic acid, (E) pO₂ and (F) pCO₂. Data are shown as mean (\pm standard error). For better visualization, error bars are directed only in one direction, above for placebo (green) and below for Nitrite (red).

Table 2
Characteristics 5 min and 15 min post CPR start, mean (\pm standard deviation).

Timepoint post CPR start	Parameter	Placebo (n = 9)	Nitrite (n = 10)	p-value
5 min	pH	7.21 (0.05)	7.21 (0.07)	0.88
	Lactate (mmol/l)	8.8 (1.9)	8.3 (1.0)	0.50
	pO ₂ (mmHg)	182 (56)	196 (69)	0.64
	pCO ₂ (mmHg)	52 (7)	53 (9)	0.70
	MAP (mmHg)	117 (30)	107 (31)	0.47
	Heart rate (bpm)	298 (32)	290 (66)	0.73
15 min	pH	7.17 (0.07)	7.20 (0.07)	0.39
	Lactate (mmol/l)	6.2 (2.3)	5.6 (1.6)	0.47
	pO ₂ (mmHg)	207 (73)	209 (86)	0.96
	pCO ₂ (mmHg)	42 (5)	41 (4)	0.56

treated groups. Due to technical reasons, histology from one rat in the placebo group (Fig. 4B) was excluded.

4. Discussion

Using a rat model of VF CA [6], we demonstrate that nitrite, administered early after ROSC, rapidly crosses the BBB and achieves levels in CSF that are approximately half of that seen simultaneously in whole blood. Nitrite therapy increases brain nitrite levels to a similar extent as other tissues. We characterized the PK of intravenous nitrite administration after VF and report on laboratory and physiologic data which speak to nitrite safety as recently noted in phase I clinical trials [28]. In contrast to our prior reports in asphyxial and KCl induced CA [26,27], we failed to see a benefit for nitrite therapy in terms of post-VF brain injury, although there was no signal for harm either.

This is the first study to measure and characterize nitrite's PK not only after CA but in any form of critical illness or brain injury. Our study clearly shows that regardless of the presence or absence (i.e. sham data) of brain injury, nitrite is able to rapidly cross the BBB to enter brain tissue and CSF after a single IV dose. CSF levels are approximately

half that of whole blood which is what one would expect plasma levels to be at this time [33] implying diffusion into CSF with minimal impediment or metabolism. This is surprising given that nitrite is an anion and is not lipophilic as are most drugs which cross the BBB. The implication is there is some form of facilitated transport assisting nitrite entry [34]. Prior studies have suggested anion exchanger 1 (AE1) as a candidate for facilitated nitrite entry into erythrocytes [35]. The lower levels of nitrite in brain tissue compared to blood/CSF imply metabolism to nitrate, S-nitrosothiols or iron-nitrosyls [10,18] though we did not assay these quantities specifically. Furthermore, the degree to which tissue nitrite increases in brain, which is comparable to heart, demonstrates that the BBB does not impede nitrite from reaching its target. Nitrite levels were lowest in liver which could be explained by extensive metabolism by mitochondria [10,14], xanthine oxidase [36], mARC-1/2 [26] and cytochrome P450 [37] systems which are present in higher quantities within this tissue type. These findings support the use of nitrite to target brain injury even in the absence of BBB breakdown as we have previously demonstrated is the case in animal models of asphyxial and potassium-chloride-induced CA [38,39]. It is also possible that in human CA, longer ischemic times and perhaps other comorbidities, that are commonly observed, may result in BBB breakdown.

The PK and safety observed for nitrite therapy after CA are interesting and corroborate our human modeling. We found that whole blood nitrite was reduced considerably in the first 5 min after dose completion. This finding explains why a two compartment PK model best fits our data and is highly suggestive of early redistribution. Prior studies have demonstrated that ischemia depletes nitrite in the blood and tissue [13,16,40]. In the setting of global ischemia (CA) one would expect systemic depletion as the tissues and blood metabolize nitrite at a steadily increasing rate as oxygen tension and pH decline [25,40]. The result is a global nitrite deficiency unique to CA which explains why nitrite dosed early after reperfusion results in high rates of redistribution to restore the global tissue deficiency present after CA. One would expect this initial rapid elimination is greater after CA than focal ischemic disease (e.g. myocardial infarction or stroke) though direct comparisons have not been made. Recent human data ($n = 120$) from

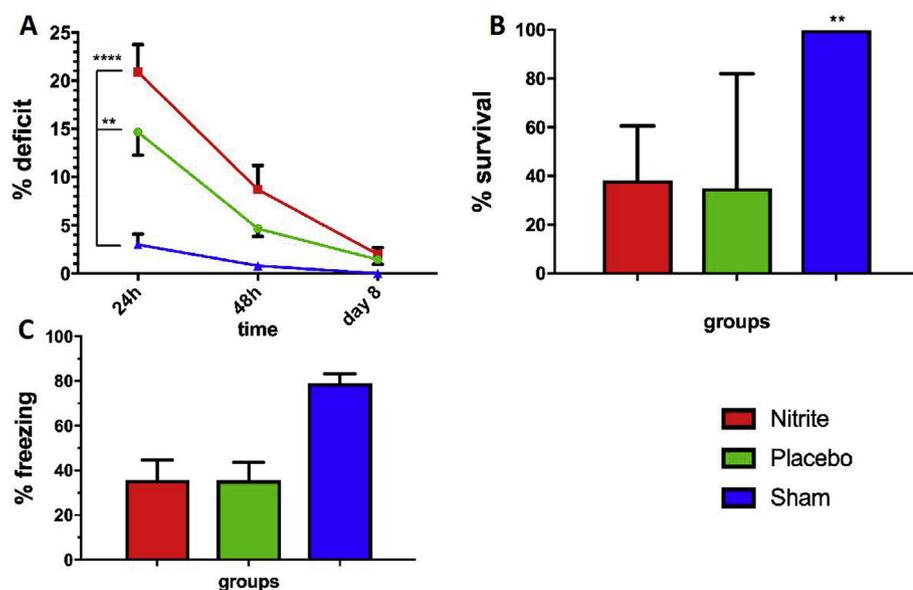


Fig. 4. Outcomes from 8-days survival model after VF CA. (A) Neurological deficit score at 24, 48 h and 8 days after CPR, (B) CA1 neuronal survival in % 8 days after CPR and (C) fear conditioning freezing on day 2 showed no significant differences between nitrite (green bar, $n = 10$) compared to placebo (red bar, $n = 9$). Sham shown as blue bar, $n = 5$. Data are shown as mean (\pm standard error).

nitrite dosing in CA support this concept of global ischemic nitrite consumption with reduced plasma levels from those anticipated based on PK models from healthy control models [29]. Since it is not feasible to perform serial blood sampling in the early post-resuscitation period after human CA, our present PK characterization provides the only data on this topic. The terminal half time for nitrite elimination which we observed (~ 30 min) is close to that noted in healthy humans (42 min) [33]. Of note the human studies were performed in healthy volunteers so the small increase in elimination rate may represent the persistent effects of global ischemia-reperfusion injury within our model which may be of relevance to ongoing human studies of nitrite after CA (NCT02987088). Since longer low and no flow ischemic times are commonly encountered in human CA compared to our animal models, the impact of both redistribution due to global nitrite depletion and terminal elimination due to persistent ischemia are likely to be even greater within human disease.

Our VF model results in significant myocardial dysfunction and shock as demonstrated by the observed lactate elevations. We recently reported this greater hemodynamic insult to be an important feature of VF compared to asphyxial CA in both rodent models and human CA [41]. Despite this we did not note any evidence of hypotension or hemodynamic instability after nitrite administration, even with peak whole blood levels of over $50 \mu\text{M}$. This is consistent with human safety data in healthy volunteers [33], patients with heart failure [42], and a large phase 1 study of nitrite dosed during CPR after out of hospital cardiac arrest, and further supports is safety with regard to hemodynamics at the doses used [29].

We observed significant brain injury within our VF CA model but did not see a neurologic improvement resulting from nitrite therapy. This contrasts to prior results in a rat model of asphyxial CA [17] and a mouse model of potassium chloride-induced CA [16] where large effects ($> 25\%$) were noted. It is possible that nitrite is less effective in VF CA, which is to say cardiac etiology CA, due to differences in the pathophysiology of injury [6] and hence response to therapy. There is precedent for this form of heterogeneous response to therapies within different models of CA [9]. Greater brain oxidative stress and mitochondrial injury [8,9] produced by asphyxia vs. VF may make the former more nitrite responsive [18]. Cerebral oxidative stress increases substantially after prolonged (> 10 min) VF [22] such that our model, with the brief CA duration used in this study, may not be sufficiently severe to capture potential benefits of nitrite therapy. It should be noted that we were not powered to detect small differences in outcome as a 5 or 10% reduction in CA1 loss would require 1470 or 367 rats per group

($\alpha = 0.05$; $1 - \beta = 0.80$), respectively. By comparison, our prior data indicated a need for $n = 6$ per group to see similar effects as previously observed. Given that in humans, CA duration is often greater than 5 min, we speculate that it is possible that benefit could be observed even in VF CA where post-arrest brain injury will exceed what we model in the lab. The ongoing nitrite clinical trial (NCT03452917) is enrolling 1500 subjects to answer this question. Since the extent of brain injury produced by VF is much less than similar durations of asphyxia [6,9] it is also possible that any benefits from nitrite against ischemia-reperfusion [10,12–18] may be offset by potential adverse effects such as increased free radical (e.g. peroxynitrite) production [43,44].

Our study has limitations. Our small sample size with a high survival rate and modest neuronal cell loss may have limited statistical power to detect a protective effect. Also, our 5-min model of VF CA might not be fully comparable to an out-of hospital cardiac arrest with long lasting CPR. Our PK characterization in VF may not be generalizable to other forms of CA or illness although our findings are close to estimates derived in healthy subjects and lung transplant recipients [29].

5. Conclusions

Using a rat model of VF CA, we characterized the PK of intravenous nitrite administration after rat VF and report safety and rapid brain penetration on par with other organs. However, contrasting studies in models of different CA phenotypes, despite showing safety, we failed to detect a benefit for nitrite therapy in reducing the modest brain injury from VF CA.

Declarations of interest

Dr. Dezfulian is the principal investigator for an investigator initiated phase 2 randomized clinical trial of inhaled nitric oxide after out-of-hospital cardiac arrest (NCT03079102) funded by Mallinckrodt Pharmaceuticals, Inc. Drs. Dezfulian, Empey and Kim are investigators in a phase 2 randomized clinical trial of nitrite after out-of-hospital cardiac arrest (NCT03452917) funded by NHLBI.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.niox.2019.09.003>.

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