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## Ascorbate Deficiency Confers Resistance to Hippocampal Neurodegeneration after Asphyxial Cardiac Arrest in Juvenile Rats

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### Abstract

**Background:** Asphyxial cardiac arrest (CA) is a significant cause of death and disability in children. Using juvenile Osteogenic disorder Shionogi (ODS) rats that, like humans, do not synthesize ascorbate, we tested the effect of ascorbate deficiency on functional and histological outcome after CA.

**Methods:** Postnatal day 16–18 milk-fed ODS and wild-type Wistar rats underwent 9-min asphyxial CA (n=8/group) or sham surgery (n=4/group). ODS mothers received ascorbate in drinking water to prevent scurvy. Levels of ascorbate and glutathione (GSH) were measured in plasma and hippocampus at baseline and after CA. Neurologic deficit score (NDS) was measured at 3, 24 and 48 hours and hippocampal neuronal counts, neurodegeneration, and microglial activation were assessed at day 7.

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#### Author Contributions

Drs. Clark, Bayır, Kochanek, Manole, and Wolf contributed to the design, and acquisition, analysis, and interpretation of data. Dr. Soysal and Ms. New contributed to data acquisition and analysis. Dr. Chen performed the animal surgeries. Dr. Wolf drafted the initial manuscript, and all authors participated in critical revision and approved this final version.

#### Disclosures

The authors do not have any relevant conflicts of interest to disclose.

**Results:** ODS rats showed depletion of plasma and hippocampal ascorbate, attenuated hippocampal neurodegeneration and microglial activation, and increased CA1 hippocampal neuron survival vs. Wistar rats while NDS were similar. Hippocampal GSH levels were higher in ODS vs. Wistar rats at baseline and 10 minutes, whereas hypoxia-inducible factor-1 $\alpha$  levels were higher in Wistar vs. ODS rats at 24 hours, after CA.

**Conclusion:** Ascorbate-deficient juvenile ODS rats appear resistant to neurodegeneration produced by asphyxia CA, possibly related to upregulation of the endogenous antioxidant GSH in brain.

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## INTRODUCTION

Cardiac arrest (CA) is a significant cause of death and neurologic disability affecting 16,000 children in the United States each year and roughly 3.3–8.0 per 100,000 worldwide (1, 2), with respiratory etiologies and asphyxia the leading cause (3). Fewer than half of children that survive have good functional outcome one year after cardiac arrest (3), with approximately one-third of survivors experiencing severe to profound functional impairment (4). Hypoxic-ischemic encephalopathy limits the functional recovery of children after CA and free radical damage caused by ischemia-reperfusion is thought to be a key contributor to hypoxic-ischemic encephalopathy.

Ascorbate (vitamin C) is an important water-soluble antioxidant. Its activity as a reducing agent mediates its role in collagen synthesis, with prolonged deficiency leading to scurvy (5). Ascorbate is concentrated via transporter proteins in neurons and glia, where it acts as a free radical scavenger (6). Compared to plasma, ascorbate levels in cerebrospinal fluid are several-fold higher owing to an active transport system in choroid plexus (7). Cerebrospinal fluid ascorbate is depleted for several days after acute traumatic brain injury (TBI) in children (8). In critically ill adults, plasma ascorbate depletion is associated with organ failure and death (9). Ascorbate levels decrease in plasma after cardiac arrest in humans (9, 10) and in the brains of rats after experimental CA (11, 12).

These findings have led to preclinical and clinical investigation into the role of ascorbate supplementation as a possible therapeutic strategy. Ascorbate treatment was shown to reduce ischemic injury in cultured cardiac myocytes (13). In patients with myocardial infarction, high-dose ascorbate was shown to improve heart function (14). Of particular relevance to our study, both intraperitoneal and intraventricular injection of ascorbic acid have been shown to reduce hypoxic-ischemic brain injury in newborn rats (15, 16). Ascorbate treatment also decreased infarct size by over 60% in a primate model of focal cerebral ischemia (17), reduced hippocampal neurodegeneration in premature fetal rabbit brain (18), and attenuated isoflurane-induced cognitive impairment in mice (19). Treatment with ascorbate was shown to increase successful resuscitation and 72-hour survival in adult rats after ventricular-fibrillation (VF)-induced CA (20). These studies have informed a clinical trial of ascorbate as a therapy after CA in adults (21).

In contrast to these promising findings, other clinical and laboratory studies have cast doubt on ascorbate's utility in acute illness. A meta-analysis of vitamin C treatment in critically ill adults failed to demonstrate an effect on mortality, acute kidney injury, or hospital length

of stay (22). Recently, two large randomized clinical trials (HYVCTTSSS and VITAMINS) did not show benefit in adult patients with septic shock treated with vitamin C, thiamine, and hydrocortisone vs. placebo (23, 24); however, a favorable association with outcome was reported in an observational study in children treated with this vitamin-drug combination (25). Relevant to CNS injury, a clinical study of vitamin C administration after ischemic stroke did not show a beneficial effect on neurological outcome (26) and vitamin C in combination with ibuprofen did not affect neurodevelopmental outcome in infants with perinatal asphyxia (27). Finally, in one study of VF-induced CA in rats, treatment with ascorbate resulted in significantly increased mortality (28).

In contrast to studies of ascorbate supplementation, there have been few studies examining the impact of ascorbate deficiency in models of brain injury, cardiac ischemia-reperfusion, or experimental CA. Ascorbate deficiency was associated with faster myocardial recovery after *in-vivo* (29) and *ex-vivo* (30) cardiac ischemia-reperfusion in rat models.

Given the conflicting clinical and preclinical evidence of its beneficial vs. detrimental effects, it is critical to gain improved understanding of ascorbate's role in CA. Prior studies of ascorbate's role as an antioxidant have generally been conducted in rodent models. Rats, along with most mammalian species, are able to synthesize ascorbate. Humans, along with other "dry-nosed" primates, fruit bats, and guinea pigs, express a non-functional version of the enzyme L-gulonolactone oxidase, a rate-limiting enzyme in ascorbate synthesis. Instead, these species rely on dietary sources. Osteogenic disorder Shionogi (ODS) rats also lack functional L-gulonolactone oxidase, and rely on dietary ascorbate (31). Therefore, ODS rats may serve as a better model for assessing the role of ascorbate in human disease.

To investigate the effects of ascorbate deficiency and the inability to synthesize ascorbate endogenously in hypoxic-ischemic encephalopathy, we used our established model of pediatric asphyxial CA (32), and studied that insult specifically in postnatal day (PND) 16–18 ODS rats. We hypothesized that juvenile ascorbate deficient ODS rats would have worse neurologic outcome after asphyxial CA.

## METHODS

Studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. We used postnatal day (PND) 16–18 male ODS rats bred on a Wistar background (33) and wild-type Wistar rats (Envigo, Indianapolis, IN). ODS rats were bred in-house and the colony maintained by the University of Pittsburgh Division of Laboratory Animal Research. Adult ODS rats were supplied with ascorbate-supplemented water (2 g ascorbate per mL, Fisher Chemical, Waltham, MA). Immature ODS rats rely on maternal milk for vitamin C supply prior to weaning, as rat milk is known to contain ascorbate (34). Pups were kept with their dams until PND 21–28.

Groups of ODS and Wistar rats were used for assessment of plasma ascorbate and glutathione (GSH), neurological deficit score (NDS) testing, immunohistochemistry, and histology. Separate groups of ODS and Wistar rats were used to assess hippocampal (and

plasma) ascorbate and GSH. Hippocampal tissue was used for biochemical studies as the CA1 region is selectively vulnerable in this model (32).

### **Animal model of asphyxial CA**

We used an established model of pediatric asphyxial CA (32) and extrapolated it to use in ODS rats. PND 16–18 male ODS or Wistar rats were anesthetized with 3.5% isoflurane/50% N<sub>2</sub>O/balance oxygen until unconscious. Rats were tracheally intubated with a 16-gauge angiocatheter and mechanically ventilated. Femoral arterial and venous catheters were placed by inguinal cut-down and insertion of PE-10 tubing and secured with sutures. Temperature, electrocardiogram, electroencephalogram, and arterial blood pressure were measured continuously. A bolus of vecuronium (1 mg/kg, IV) was given five minutes before asphyxia to prevent spontaneous breathing. Isoflurane was discontinued and FiO<sub>2</sub> was reduced to 0.21 two minutes before asphyxia for anesthetic washout and to eliminate hyperoxemia, respectively. Asphyxia was induced by disconnecting the ventilator from the tracheal tube for 9 minutes. Rats were then resuscitated by resumption of mechanical ventilation, administration of epinephrine (0.005 mg/kg, IV) and sodium bicarbonate (1 mEq/kg, IV), and initiation of chest compressions until return of spontaneous circulation (ROSC). Upon resumption of spontaneous breathing, rats were extubated and returned to their cages. Sham rats were anesthetized, intubated, and had femoral arterial and venous catheters placed, but did not undergo asphyxial CA.

### **Measurement of ascorbate in plasma and hippocampus**

Rats were sacrificed at 10 minutes or 24 hours after asphyxial CA and ROSC and 24 hours after sham injury (n = 5 per group). Blood samples were obtained at baseline from the femoral arterial catheter and at time of sacrifice by cardiac puncture after euthanasia under isoflurane anesthesia. All samples were placed in ethylenediaminetetraacetic acid (EDTA)-containing blood collection tubes (Becton Dickinson, Franklin Lakes, NJ), protected from light. Blood was centrifuged at 1,000 ×g for 5 minutes at 4°C to separate plasma. Meta-phosphoric acid was added to plasma specimens (final concentration 5%, Sigma-Aldrich, St. Louis, MO) to preserve ascorbate.

After phlebotomy rats were transcatheterially perfused with ice-cold heparinized saline. Brains were quickly dissected on ice. The left hippocampus was extracted and frozen in liquid nitrogen. Hippocampi were homogenized with a tissue-tearor in lysis buffer (1% Triton X-100, pH 7.4). A portion of this homogenate was reserved for determination of protein concentration, and a portion was treated with meta-phosphoric acid (final concentration 5%).

Ascorbate was measured using a high-throughput assay based on the direct reaction of ascorbate with a nitroxide radical conjugated to a fluorescent acridine moiety (4-((9-acridinecarbonyl)-amino)-2,2,6,6-trimethylpiperidine-1-oxyl radical, AC-TEMPO), yielding a fluorescent hydroxylamine product (AC-TEMPO-H) as described by Belikova et al. (35). Ascorbate levels are reported in units of nmol/mL for plasma samples, and nmol/mg protein for hippocampal homogenates.

### **GSH assay in plasma and hippocampus**

In the same rats used for ascorbate assays ( $n = 5$  per group), GSH assays were performed as described previously by Bayir et al. (8, 36), based on the reagent ThioGlo-1 (EMD Millipore., Bedford, MA), which produces a fluorescent product upon its reaction with sulfhydryl groups. Plasma samples and hippocampus homogenates obtained as described above were treated with EDTA and protease inhibitor (Halt Protease Inhibitor Cocktail, Thermo Fisher Scientific, Waltham, MA). GSH concentrations in hippocampus were determined by addition of GSH peroxidase and hydrogen peroxide (Sigma, St. Louis, MO) to the brain homogenates, and the resultant fluorescence response was subtracted from the fluorescence response of the same specimens without addition of GSH peroxidase and hydrogen peroxide. A spectrophotometer (Filtermax F5, Molecular Devices, LLC, San Jose, CA) was employed using 388nm (excitation) and 500nm (emission) wavelengths. GSH levels are reported as of nmol/mL for plasma samples, and nmol/mg protein for hippocampal homogenates

### **Functional outcome assessment**

We assessed functional outcome after CA using NDS (37) adapted for use in juvenile rats at 3, 24, and 48 hours after asphyxial CA and ROSC ( $n = 8$ /group) or sham injury ( $n = 4$ /group). This score evaluates the rats' general appearance, cranial nerve, motor, and sensory function, as well as coordination on a scale from 0–500, with higher scores representing worse function. Level of consciousness and respiratory pattern were each graded from 0–100, for a total of 200. Cranial nerve function was assessed by testing olfactory, visual, and auditory responses, corneal reflex, and whisker motion to stimulation on a scale of 20 points each for a total of 100 points. Sensory and motor function in each paw and the tail were tested on a scale of 10 points each, for a total of 100 points, respectively. Coordination was evaluated by observing rats traverse a travel ledge, stop at a table edge, and demonstrate placing, righting reflexes. Total score was divided by 5 to yield percent NDS. A score of 0 indicates no dysfunction, whereas a score of 100% indicates brain death.

### **CA1 hippocampal neurodegeneration and microglial immunohistochemistry**

After completion of NDS testing and seven days after CA ( $n = 8$ /group) or sham injury ( $n = 4$ /group), rats were transcardially perfused with ice-cold heparinized saline followed by 10% formalin. Brains were removed and fixed in 10% formalin, dehydrated in a series of ethanol solutions and xylene, and embedded in paraffin. Coronal sections (5- $\mu$ m thickness) were cut at the level of the dorsal hippocampus using a microtome. The sections were further processed with hematoxylin and eosin (H&E) for hippocampal neuron counting and Fluor Jade B (FJB; Millipore, Burlington, MA) to evaluate neurodegeneration as described (32). Microglial activation was assessed by Iba1 immunostaining (1:250 dilution; Wako Chemicals, Richmond, VA) using a biotin-conjugated anti-rabbit secondary antibody, developed with avidin-conjugated horseradish peroxidase (ABC Elite Kit, Vector Laboratories, Burlingame, CA), and counterstained with hematoxylin as described (38).

Neurons and microglial cells in hippocampal area CA1 were counted in coronal sections by an observer blinded to study group. At 10 $\times$  magnification, hippocampal area CA1 was identified. For H&E-stained slides, the proportion of neurons showing ischemic changes

(i.e. eosinophilia, compaction) in CA1 hippocampus was recorded. For FJB slides, the total number of FJB-positive neurons per mm of CA1 was recorded. To quantify microglial activation, the total number of Iba1-positive cells per high-power field was recorded.

### Western blot analysis

Western blot analysis for HIF-1 $\alpha$  was performed using methods described previously (38). Briefly, protease inhibitors were added to hippocampal homogenates (n = 4/group) used for ascorbate and GSH assays. Protein concentration was determined by bicinchoninic acid assay and 35  $\mu$ g protein aliquots were loaded onto pre-cast TGX 4–20% gels (BioRad, Hercules, CA), separated electrophoretically, then transferred onto polyvinylidene difluoride membranes (Millipore Immobilon, Bedford, MA). Membranes were incubated with a rabbit monoclonal antibody against HIF-1 $\alpha$  (1:800 dilution; [EPR16897] ab179483; Abcam, Cambridge, MA) overnight at 4°C then appropriate secondary antibody for 2 hours at room temperature. Protein bands were visualized with Supersignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL) and imaged on a BioRad Chemi Doc XRS+ imaging system with band intensity analyzed by ImageLab Software (Version 4.0, BioRad). Membranes were stripped and re-probed with antibody against  $\beta$ -actin (1:2,000) using the methods above. HIF-1 $\alpha$  densitometry measurements were normalized to corresponding actin densitometry measurements within each lane. Fold-increase in HIF-1 $\alpha$  was determined using corresponding group-specific shams from the same membrane.

### Statistical analysis

Data were analyzed with the statistical software Prism 7.0c (GraphPad Software Inc., La Jolla, CA). Data are presented as mean  $\pm$  standard error (SEM). Mean baseline weights between groups were compared using an unpaired student's t-test. Serial physiologic data between groups were compared using two-way repeated measures (RM) ANOVA with Šídák's or Tukey's post hoc test for multiple comparisons. Physiologic data between sham ODS and Wistar rats, and biochemical and histological data between groups, were compared using two-way ANOVA with Šídák's post hoc test. NDS between groups were compared using two-way RM ANOVA with Tukey's post-hoc test for multiple comparisons. A  $p < 0.05$  was considered statistically significant.

## RESULTS

ODS rats weighed less than Wistar rats on PND 16–18 (n = 27/group,  $31.6 \pm 0.5$  vs.  $39.9 \pm 1.0$  g,  $p < 0.001$ ). Two ODS rats died before the designated sacrifice time after CA and were replaced. Table 1 shows the physiological variables of rats prior to asphyxial CA (baseline), and then at 10, 30, and 60 minutes after ROSC. Temperature, pH, PaCO<sub>2</sub>, and PaO<sub>2</sub> levels were not different between ODS and Wistar CA groups at all timepoints. ODS vs. Wistar rats had lower mean arterial blood pressure at baseline (n = 18/CA group, 9/sham group,  $56 \pm 2$  vs.  $63 \pm 2$ ,  $p < 0.05$ ) and at 10 minutes after ROSC ( $48 \pm 2$  vs.  $65 \pm 3$ ,  $p < 0.05$ ), and lower arterial lactate levels at 10 minutes after ROSC ( $6.0 \pm 0.1$  vs.  $7.0 \pm 0.3$ ,  $p < 0.05$ ). Baseline temperature, pH, PaCO<sub>2</sub>, PaO<sub>2</sub>, and lactate levels were similar between sham ODS and Wistar rats.

### Ascorbate levels in plasma and hippocampus in ODS vs. Wistar rats

Plasma ascorbate levels (Figure 1A) were lower in ODS vs. Wistar rats at baseline (n = 14–16,  $5.9 \pm 1.3$  vs  $34.7 \pm 2.4$  nmol/mL,  $p < 0.0001$ ), 10 minutes (n = 5/group,  $4.3 \pm 1.3$  vs  $41.3 \pm 9.0$ ,  $p < 0.0001$ ) and 24 hours ( $5.0 \pm 3.0$  vs  $25.0 \pm 8.0$  nmol/mL,  $p < 0.001$ ) after ROSC, and 24 hours after sham injury (n = 5/group,  $2.7 \pm 1.7$  vs.  $34.4 \pm 4.7$  nmol/mL,  $p < 0.0001$ ). Hippocampal ascorbate levels (Figure 1B) were also lower in ODS vs. Wistar rats 10 minutes after ROSC (n = 5/group,  $25.4 \pm 0.9$  vs  $44.0 \pm 3.0$  nmol/mg protein,  $p < 0.05$ ) and 24 hours after sham injury (n = 5/group,  $25.7 \pm 1.2$  vs.  $44.7 \pm 7.3$  nmol/mg protein,  $p < 0.02$ ). At 24 hours after CA, hippocampal ascorbate levels were similar in ODS vs. Wistar rats (n = 5/group,  $24.8 \pm 1.6$  vs.  $38.0 \pm 7.2$  nmol/mg protein,  $p = 0.14$ ).

### GSH levels in plasma and hippocampus in ODS vs. Wistar rats

Plasma GSH levels (Figure 2A) were lower in ODS vs. Wistar rats at 24 hours after CA (n = 5/group,  $238.1 \pm 7.6$  vs.  $281.3 \pm 8.5$  nmol/mL,  $p < 0.05$ ). Plasma GSH levels were similar between ODS and Wistar rats after sham injury and at 10 minutes after ROSC. Hippocampal GSH levels (Figure 2B) were higher in ODS vs. Wistar rats at 10 minutes after ROSC (n = 5/group,  $27.9 \pm 1.5$  vs.  $22.9 \pm 1.6$  nmol/mg protein,  $p < 0.05$ ) and after sham injury (n = 5/group,  $29.5 \pm 1.0$  vs.  $23.6 \pm 1.1$ , nmol/mg protein,  $p < 0.05$ ).

### NDS in ODS and Wistar rats

Rats demonstrated moderate to severe functional impairment 3 hours after ROSC, with ODS and Wistar rats having similar 3-hour NDS (n = 8/group,  $64 \pm 2.7\%$  vs.  $70 \pm 2.6\%$ ,  $p = 0.12$ ). Both ODS and Wistar rats had higher NDS at 3-hr after ROSC than their respective sham groups (n = 4/group,  $10 \pm 7\%$  vs.  $64 \pm 3\%$ , ODS sham vs. ODS CA  $p < 0.0001$ , and  $2 \pm 0.4\%$  vs.  $70 \pm 3\%$ , Wistar sham vs. Wistar CA,  $p < 0.0001$ ). At 24 and 48 hours after CA, rats in both groups had NDS similar to their respective sham groups. NDS were similar between ODS and Wistar at all timepoints (Figure 3).

### CA1 hippocampal neurodegeneration and microglial activation in ODS vs. Wistar rats

ODS rats showed attenuated neurodegeneration in hippocampal area CA1 compared with Wistar rats (Figure 4). A lower percentage of ischemic neurons identified as eosinophilic and shrunken by H&E staining was seen in ODS vs. Wistar rats 7 days after CA (n = 8/group,  $7 \pm 2$  vs.  $19 \pm 5\%$ ,  $p < 0.05$ ). Neurodegeneration detected using FJB was also reduced in ODS vs. Wistar rats 7 days after CA ( $16 \pm 4$  vs.  $46 \pm 12$ /mm,  $p < 0.05$ ). Finally, microglial activation, identified by Iba1 immunoreactivity, was attenuated in ODS vs. Wistar rats within hippocampal area CA1 ( $25 \pm 4$  vs  $62 \pm 10$ /high-power field).

### Relative HIF-1 $\alpha$ abundance in hippocampal samples from ODS vs. Wistar rats

Figure 5 shows western blots for HIF-1 $\alpha$  in hippocampal samples from sham rats and rats 10 min and 24 hours after CA (n = 4/group). In Wistar rats, the relative abundance of HIF-1 $\alpha$  was increased 24 hours after CA compared with Wistar sham ( $p < 0.05$ ). There was no difference 10 min after CA vs. sham ( $p > 0.96$ ). In ODS rats, there was no difference in the relative abundance of HIF-1 $\alpha$  at 10 min or 24 hours compared with ODS sham ( $p > 0.99$  for both). Wistar rats 24 hours after CA had a  $4.0 \pm 1.0$ -fold increase in relative HIF-1 $\alpha$ .

abundance compared with ODS rats 24 hours after CA where a  $1.4 \pm 0.5$ -fold increase was observed ( $p < 0.05$ ).

## DISCUSSION

To our knowledge, this is the first report of the effects of ascorbate deficiency on neurodegeneration in a pre-clinical model of pediatric asphyxial CA. Contrary to our initial hypothesis, our data show that ascorbate deficient ODS rats are resistant to neurodegeneration and microglial activation after asphyxial CA. Ascorbate deficiency in ODS rats was verified in plasma and hippocampal tissue. While dams received vitamin C supplementation in drinking water, this did not translate to normal ascorbate levels in breastfeeding pups (Figure 1). To begin to address the mechanism underlying this unanticipated finding, we found that hippocampal levels of GSH were higher in ascorbate deficient ODS vs. Wistar rats on PND 16–18 (Figure 2), supporting the possibility of upregulation of this important intracellular antioxidant in the face of low circulating ascorbate in pre-weanling ODS rats. Caution is in order, however, given the relatively small sample sizes used to detect statistically significant differences ( $n = 5$ /group).

Normal physiological levels of plasma ascorbate in humans are  $>23$  nmol/ml, and ascorbate levels  $<11$  nmol/ml are considered deficient (39). PND 16–18 ODS rat pups used in our study had baseline plasma ascorbate levels of approximately 6 nmol/mL but did not display evidence of scurvy, although they weighed less and had shorter limbs than their Wistar counterparts. These levels are comparable to those originally reported in ascorbate deficient ODS rats (40). Our ODS rat pups received dietary vitamin C via milk from supplemented ODS dams. Though the level of ascorbate in the milk of ODS rats was not measured and has not been previously reported by others, non-ODS rat milk is reported to have an ascorbate concentration of 0.4 mg/100 mL (2.27 nmol/mL) (34). We observed baseline plasma ascorbate levels in Wistar rat pups that were roughly 40% higher than levels previously reported in adult Wistar rats (30).

Potential mechanisms by which ascorbate deficiency could protect neurons after asphyxial CA include upregulation of other endogenous antioxidant or neuroprotective pathways, or deleterious effects of ascorbate and/or ascorbate free radical. Our observation that ascorbate deficiency is protective in PND 16–18 ODS rats is consistent with studies by Vergely et al. in cardiac ischemia-reperfusion. In an isolated heart model of ischemia-reperfusion they found that hearts from ODS rats recovered contractility sooner than hearts from Wistar rats, with no difference in free radical release during reperfusion (30). In an *in vivo* study by the same group of investigators, Wistar and deficient ODS rats showed no difference in plasma antioxidant capacity or severe arrhythmia following regional myocardial ischemia-reperfusion (29). When adult ODS rats were subjected to stepwise decline in dietary ascorbic acid, levels of GSH in the liver and kidney remain unchanged, although brain GSH levels were not reported (41). We found that tissue levels of GSH in the hippocampus, a selectively vulnerable region in this model (32), were higher in PND 16–18 ascorbate depleted ODS vs. Wistar rats. Taken together, these studies indicate that antioxidant pathways other than ascorbate, including GSH, may be upregulated in the brain and heart in ascorbate-deficient rats possibly contributing to the reduction in hippocampal

neurodegeneration observed in this study. Teleologically this would make sense for humans and other species unable to endogenously synthesize ascorbate during times of dietary Vitamin C sparsity.

It is also possible that oxidative signaling pathways leading to increased neurotoxicity are attenuated in the setting of ascorbate deficiency. We found that hippocampal HIF-1 $\alpha$  levels are increased in Wistar rats, but not in ascorbate deficient ODS rats, 24 hours after CA. Taken together with the finding that hippocampal GSH levels are increased in ascorbate deficient ODS rats, ascorbate deficiency via upregulation of compensatory antioxidant pathways may attenuate downstream oxidative signaling pathways including HIF-1 $\alpha$ . While increased HIF-1 $\alpha$  has been shown to be neurotoxic in experimental models of cerebral ischemia (42), traumatic brain injury (43), and stroke (44), HIF-1 $\alpha$  activation has also been associated with neuroprotective effects after cerebral hypoxia-ischemia (45, 46). Thus, it is well beyond the scope of this study to speculate as to the role of HIF-1 $\alpha$  in ascorbate depleted rats after CA.

An alternative explanation for the apparent neuroprotective effect of ascorbate deficiency is the possible negative effect of ascorbate itself. In a rabbit myocardial ischemia-reperfusion model, vitamin C negated the protective effects of ischemic preconditioning (47). Ascorbate's pro-oxidant characteristics provide one potential explanation for this effect. In the presence of ferric iron, ascorbate is oxidized to form ascorbyl radical. Levels of ascorbyl radical are increased in the reperfusion phase of experimental oxygen-glucose deprivation (48). Human fibroblasts undergo NF- $\kappa$ B-mediated apoptosis when exposed to iron and ascorbate (49). In light of the observed increase in iron content of brain tissue after experimental cardiac arrest (50), this mechanism might partially explain our findings. These findings are corroborated by the observation that supplementation of healthy human subjects with iron and ascorbate results in increased DNA damage (51). Recycling of oxidized ascorbate to ascorbate in mitochondria depends on GSH (52), and we observed higher levels of ascorbate and lower levels of GSH in hippocampi from Wistar vs. ODS sham rats and 10 min after ROSC (Figures 1B and 2B).

The effects of ascorbate after ischemic injury may be dose-dependent. Experiments showing impaired resuscitation after VF-induced CA in rats utilized very high doses of ascorbate, resulting in plasma levels greater than 11,000 nmol/mL, 300-fold higher than those we measured in Wistar rats (28). In contrast, the patients treated with ascorbate after myocardial infarction had plasma levels of 100 nmol/mL (14). Concentration-dependent effects of ascorbate may partially explain the discordant conclusions of prior studies.

Ascorbate deficiency may also lead to neuroprotection via differential transport of vitamin C. In the developing brain, the sodium-dependent vitamin C transporter-2 (SVCT2) is up-regulated, allowing for concentration of ascorbate in brain tissue (6). SVCT2 transcription increases in the brain after experimental focal cerebral ischemia (53), and in cultured hippocampal cells, SVCT2 knockout increases susceptibility to oxidant damage from excitotoxicity (54). Notably, we observed smaller differences in ascorbate concentration in brain than in plasma in PND 16–18 ODS rats. The potential role of SVCT2 expression in ODS rats after CA warrants further study.

Our study has limitations. First, NDS was used to evaluate gross motor deficits early after CA. Further studies using more advanced behavioral outcomes at later timepoints in both ascorbate deplete and ascorbate replete ODS rats after CA appears warranted. Second, PND 16–18 ODS rats weighed less, had a small difference in plasma lactate at 10 minutes after ROSC, and had lower mean arterial blood pressure at baseline and 10 minutes after ROSC vs. their Wistar counterparts (Table 1). However, mean arterial pressure values fall within the autoregulatory range for rats (55), and if anything lower blood pressure after CA would be anticipated to bias toward more neurodegeneration. That said, it will be important to evaluate the systemic effects as well as effects on other organs, particularly the heart and kidney, of both pre- and post-injury ascorbate depletion in future studies. Third, while plasma and brain ascorbate and GSH were measured, a comprehensive evaluation of antioxidant reserves and response to oxidative stress was beyond the scope of the present study. Future study is warranted to delineate the mechanism underlying the neuroprotective effects observed in ascorbate depleted ODS rats after CA. This should include experiments to determine whether withholding dietary ascorbate post-insult, mimicking the clinical scenario, is protective, detrimental or neither after severe CA; and clinical studies to determine whether ascorbate levels and compensatory antioxidant pathways on admission are associated with outcome in children suffering CA.

## Conclusion

Ascorbate deficient juvenile ODS rats display neuroprotection after experimental asphyxial CA compared with Wistar rats. Possible explanations for this resiliency include upregulation of other antioxidant pathways including the GSH pathway, attenuated activation of downstream oxidative signaling pathways, and/or potential harmful effects of ascorbate and/or ascorbyl free radical, given the capacity of mammals to synthesize ascorbate *de novo*. Additional study is warranted to further develop the experimental ODS model for application to human conditions where ascorbate appears to play a key mechanistic role, such as hypoxia-ischemia in the developing brain.

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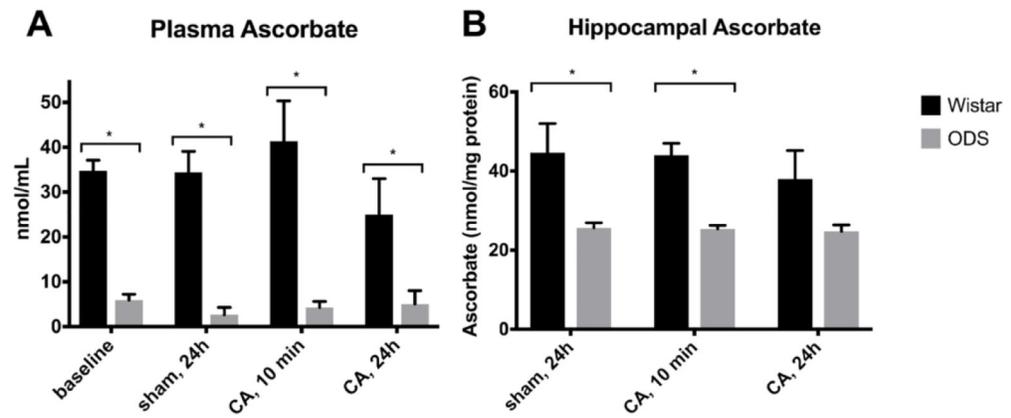
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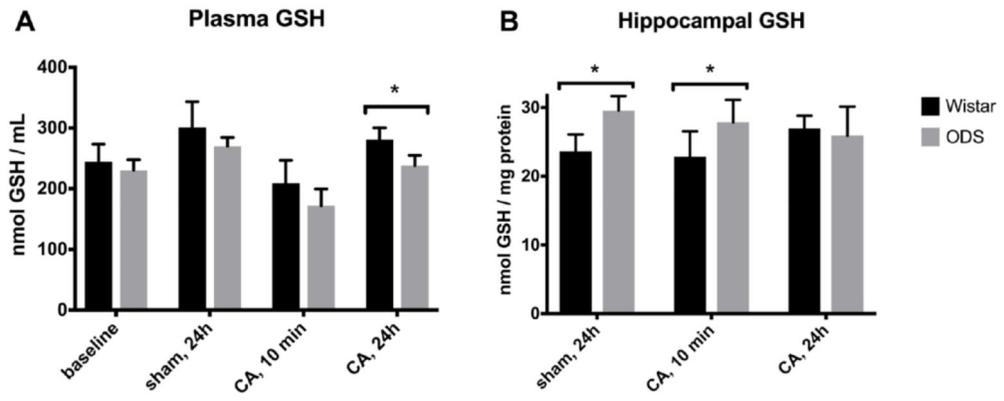
**Impact Statement**

- Like humans and unlike other rodents, osteogenic disorder Shionogi (ODS) rats do not synthesize ascorbate, and thus may serve as a useful model for studying the role of ascorbate in human disease.
- Conflicting evidence exists regarding ascorbate's protective versus detrimental effects in animal models and clinical studies.
- Ascorbate-deficient ODS rats are resistant to neurodegeneration after experimental cardiac arrest.

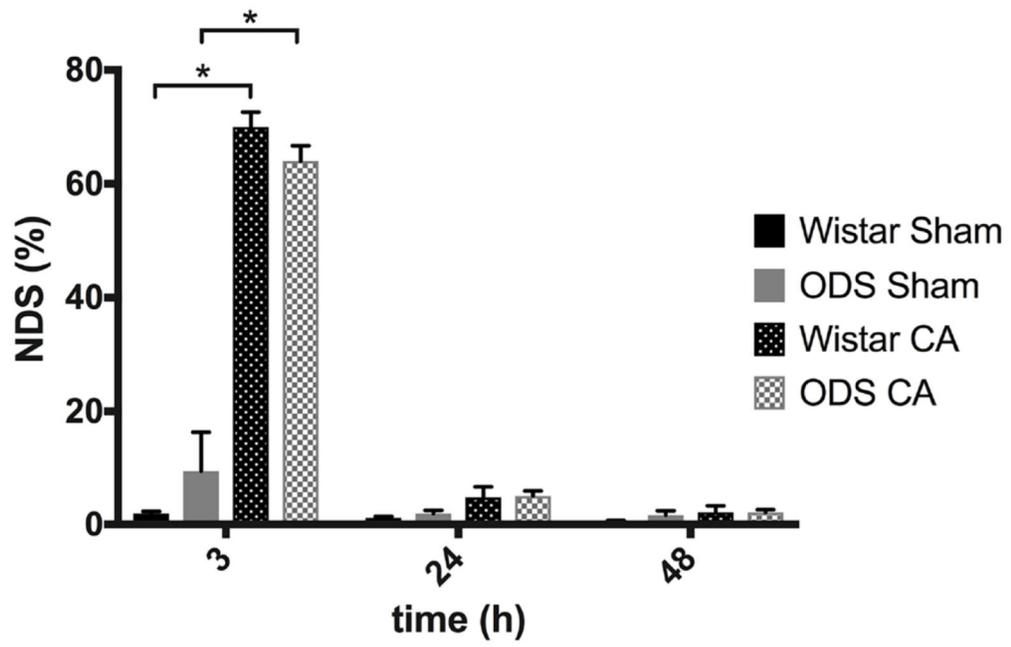


**Figure 1.**

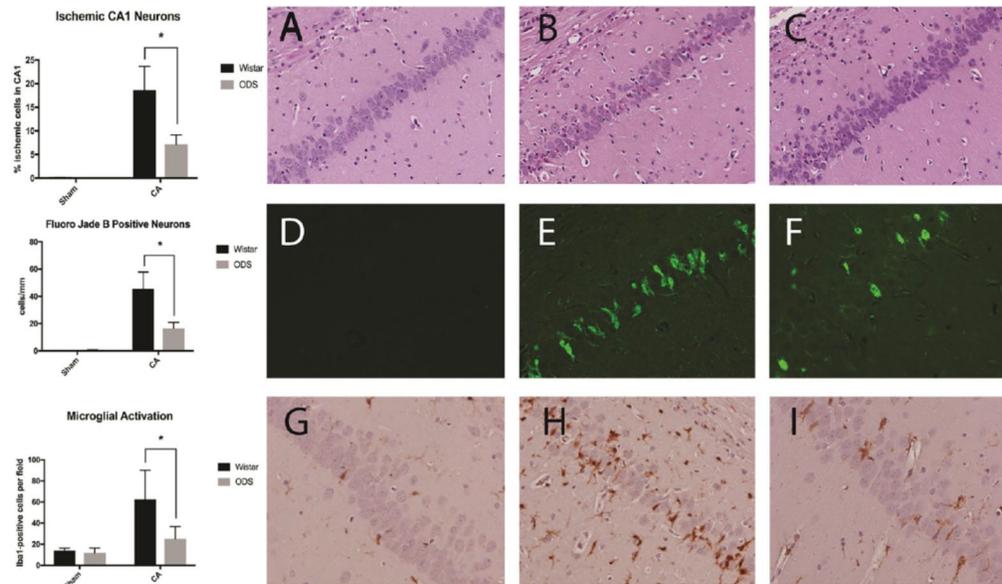
A. Ascorbate levels in plasma at baseline, 10 min and 24 h after ROSC, and 24 h after sham surgery. B. Ascorbate levels in hippocampus at 10 min and 24 h after ROSC, and 24 h after sham surgery. \* $p < 0.05$  vs. Wistar rats, ANOVA; baseline,  $n = 15$ /group; sham, 10 min CA, and 24 h CA,  $n = 5$ /group; mean  $\pm$  SEM.



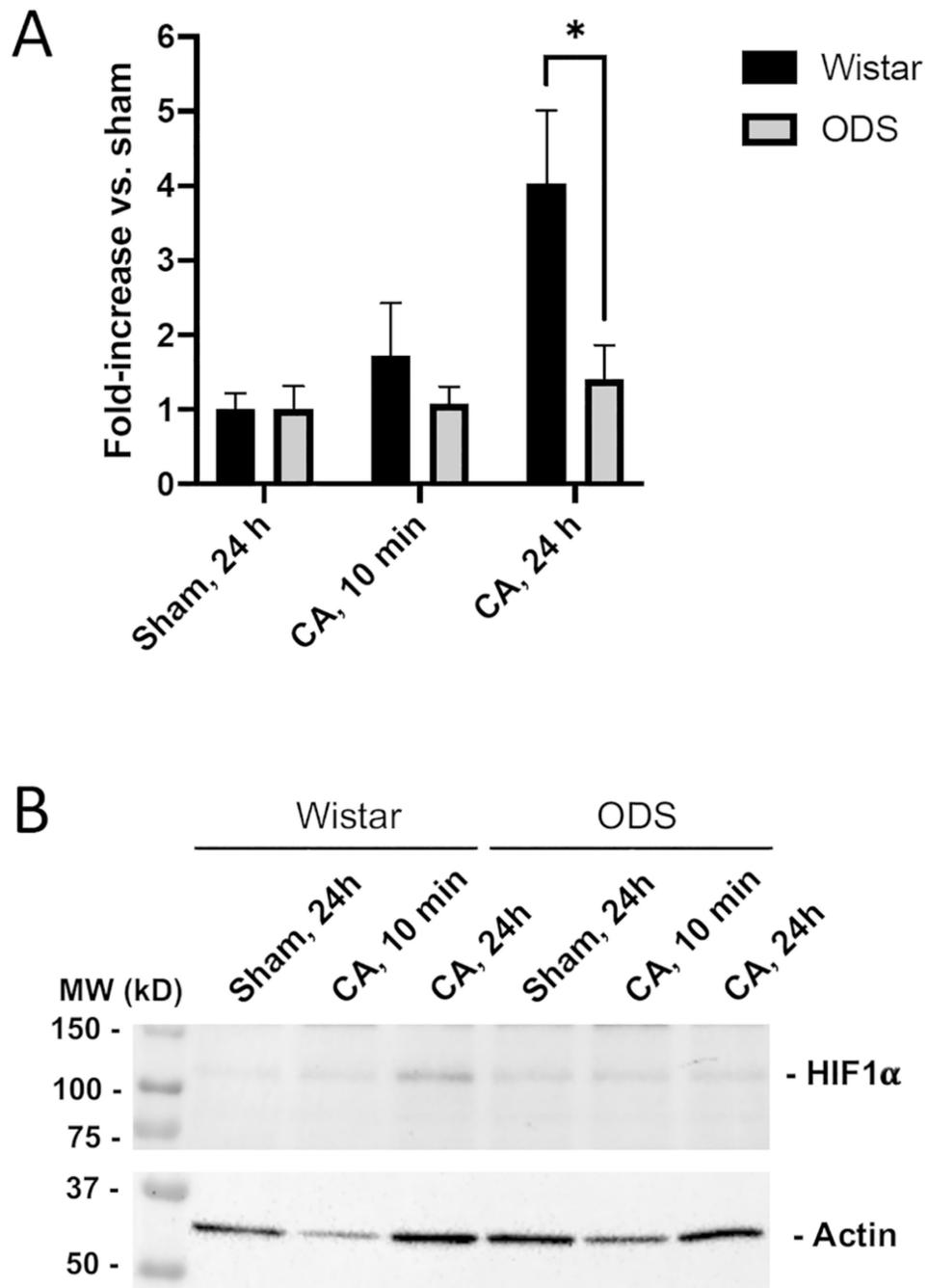
**Figure 2.** GSH levels in plasma at baseline, 10 min and 24 h after ROSC, and 24 h after sham surgery. B. GSH levels in hippocampus at 10 min and 24 h after ROSC, and 24 h after sham surgery. \*p < 0.05, ANOVA; baseline, n = 15/group; sham, 10 min CA, and 24 h CA, n = 5/group; mean ± SEM.



**Figure 3.** NDS 3, 24, and 48 h after ROSC or sham surgery. \* $p < 0.05$ , ANOVA; CA,  $n = 8$ /group; sham,  $n = 4$ /group; mean  $\pm$  SEM.



**Figure 4.** Immunohistochemical and histologic assessment of CA1 hippocampus 7 d after asphyxial CA or sham surgery. Top row: H&E-stained sections taken from sham (A), Wistar CA (B), and ODS CA (C). Middle row: FJB-treated sections taken from sham (D), Wistar CA (E), and ODS CA (F). Bottom row: Iba1 immunostained sections taken from sham (G), Wistar CA (H), and ODS CA (I). \* $p < 0.05$ , ANOVA; CA,  $n = 8$ /group; sham,  $n = 4$ /group; mean  $\pm$  SEM.



**Figure 5.** A. Relative HIF-1 $\alpha$  abundance reported as fold-increase vs. corresponding sham control in hippocampal homogenates after sham surgery and 10 minutes and 24 hours after asphyxial CA (\* $p < 0.05$ ,  $n = 4/\text{group}$ , mean  $\pm$  SEM). B. Representative western blots for HIF-1 $\alpha$  and actin (loading control).

**Table 1.**

Physiological variables

	ODS Rats				
	Sham	Baseline	10 min	30 min	60 min
<b>T (°C)</b>	37.0±0.13	36.8±0.07	36.8±0.07	36.9±0.07	37.0±0.06
<b>MAP (mmHg)</b>	56±8	56±2	48±2	42±2	45±1
<b>pH</b>	7.33±0.03	7.31±0.01	7.20±0.02	7.36±0.02	7.43±0.01
<b>P<sub>a</sub>CO<sub>2</sub> (mmHg)</b>	39±2	42±2	40±2	37±1	39±1
<b>P<sub>a</sub>O<sub>2</sub> (mmHg)</b>	235±8	209±11	389±14	370±31	406±14
<b>Lactate (mmol/L)</b>	0.8±0.08	0.9±0.12	6.0±0.14	2.5±0.18	0.6±0.04
	Wistar Rats				
	Sham	Baseline	10 min	30 min	60 min
<b>T (°C)</b>	37.0±0.09	37.0±0.12	37.0±0.12	36.8±0.07	37.0±0.09
<b>MAP (mmHg)</b>	64±1	63±2 <sup>*</sup>	65±3 <sup>*</sup>	43±2	47±2
<b>pH</b>	7.39±0.01	7.36±0.01	7.20±0.03	7.38±0.01	7.41±0.01
<b>PaCO<sub>2</sub> (mmHg)</b>	38±1	39±2	46±3	41±1	39±2
<b>PaO<sub>2</sub> (mmHg)</b>	243±5	208±18	391±28	422±16	442±11
<b>Lactate (mmol/L)</b>	0.8±0.08	1.2±0.07	7.0±0.31 <sup>*</sup>	2.4±0.16	0.7±0.06

*Abbreviations:* MAP, mean arterial blood pressure; ODS, osteogenic disorder Shionogi; PaCO<sub>2</sub>, partial pressure of carbon dioxide; PaO<sub>2</sub>, partial pressure of oxygen; T, temperature.

n = 4/group, sham

n = 7–8/group, Baseline, 10 min, 30 min, 60 min

<sup>\*</sup> *p* < 0.05 vs. ODS rats., mean ± SEM, RM ANOVA