

Glutathione and Ascorbic Acid Enhance Recovery of Guinea Pig Spinal Cord White Matter Following Ischemia and Acrolein Exposure

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Key Words

Neurotrauma · Oxygen glucose deprivation · Reperfusion · Membrane · Antioxidants

Abstract

Objective: We have shown that acrolein, a lipid peroxidation byproduct, can inflict significant damage in isolated spinal cord white matter following oxygen glucose deprivation (OGD). The mechanism of such acrolein-induced damage is unclear. The aim of this study was to examine whether glutathione (GSH) and ascorbic acid, two reactive oxygen species (ROS) scavengers, can alleviate functional and anatomical damage due to acrolein.

Methods: We used an OGD injury model with isolated guinea pig spinal cord white matter. Sucrose gap recording was used to monitor axonal impulse conduction, and a horseradish peroxidase exclusion test was employed to determine membrane integrity. The functional and anatomical parameters were compared in three groups: acrolein, acrolein/GSH and acrolein/ascorbic acid. **Results:** We found that while GSH resulted in an 87% recovery of compound action potential conductance, ascorbic acid produced a 97% recovery, compared with a 69% recovery in an injured group without treatment. It is noted that GSH, and to a lesser extent ascorbic acid, preferentially enhanced functional recovery in smaller ax-

ons. **Conclusion:** Acrolein-induced neuronal damage is likely mediated by ROS. Furthermore, GSH and ascorbic acid are effective in suppressing acrolein and free radical-induced injury in spinal cord white matter.

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Introduction

It is well established that ischemia, as a primary or secondary injury, could trigger numerous debilitating effects leading to functional paralysis [1–5]. Despite the obvious and severe consequences of ischemic injury, the exact mechanism of ischemia-mediated detriment is not clear. This is particularly true in in vivo injuries where various factors are involved. The effort to break down this cascade and preserve damaged tissue is hampered by the lack of understanding of the key pathology in such injury.

In order to dissect out the potential roles of the many concomitant factors that are triggered by ischemia, we employed a well-controlled in vitro spinal cord oxygen glucose deprivation (OGD) model. In this model, many factors can be eliminated and reintroduced separately or in combination to control the experimental situation [6]. With this model, we have found that when other factors are controlled, a 60-min OGD alone did not inflict long-term functional or anatomical deficits [6, 7]. However,

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when acrolein, a lipid peroxidation (LPO) byproduct which is known to be elevated following spinal cord injury [8], was introduced to the injury, compounding OGD, significant functional and anatomical damage resulted [7]. Interestingly, acrolein at the same concentration (50 μ M) did not cause any short-term damage when applied without OGD [9], indicating a synergistic detrimental effect of OGD and acrolein. Furthermore, we have noted that acrolein can increase the level of reactive oxygen species (ROS) and LPO in control uninjured spinal cord tissue and that the acrolein-mediated functional and anatomical damage is mostly dependent on elevated levels of ROS in such condition [10]. Based on these findings, we hypothesize that acrolein plays a critical role in ischemic injury by stimulating the level of LPO and ROS which subsequently inflicts various cellular destructions. Consistent with this idea, ROS and LPO have been found to be elevated following ischemia [11–13], as well as after traumatic injury [14, 15].

There are many endogenous free radical scavenger systems which offer protection against oxidative stress [16, 17]. However, during a more complex injury, these endogenous resistance systems are usually overwhelmed and compromised, which contributes to the overall uncontrollable elevation of ROS and related injuries [14, 15, 18]. Hence, enhancing endogenous antioxidant systems such as glutathione (GSH) by supplying the precursors of GSH (γ -glutamylcysteine) [19] or stimulating the enzyme that produces GSH [20] are thought to be effective treatments to curtail ROS-related injuries [21]. Similar to GSH, ascorbic acid is also an effective antioxidant in vitro and has been shown to effectively scavenge ROS in vivo as well [16, 22, 23]. However, the value of ascorbic acid as an effective treatment for oxidative stress has been controversial [22, 24].

The current investigation was designed to examine the protective effects of GSH and ascorbic acid on the functional and anatomical recovery in an in vitro ischemic model. This study is a logical step to test our hypothesis concerning the mechanism of acrolein-mediated damage by attempting to alleviate such injury with ROS scavengers. This study is expected to further clarify the role of ROS in acrolein-mediated damage in this in vitro OGD injury. Further, it will also offer insights into the functional and anatomical significance of suppressing ROS and acrolein in ischemic insults. The exploration of these factors will likely help us design effective interventions for acute spinal cord trauma and, possibly, chronic neurodegenerative diseases.

Experimental Procedures

Isolation of Spinal Cord White Matter

Adult female Hartley guinea pigs of 350–500 g body weight were used. They were deeply anesthetized with a combination of ketamine (80 mg/kg) and xylazine (12 mg/kg). They were then perfused through the heart with cold (15°C) oxygenated Krebs' solution to remove the blood and lower core temperature. The entire vertebral column was excised rapidly and a complete laminectomy was performed. The spinal cord was removed from the vertebrae and immersed in cold Krebs' solution and immediately subdivided, first along the sagittal midline and then by cutting each half of the cord radially in order to isolate the ventral white matter. Each white matter strip was subsequently incubated for 1 h in fresh Krebs' solution bubbled continuously with 95% oxygen/5% carbon dioxide at room temperature. The composition of the Krebs' solution was as follows (in mM): 124 NaCl, 5 KCl, 1.2 K₂HPO₄, 1.3 MgSO₄, 2 CaCl₂, 20 glucose and 26 NaHCO₃, equilibrated with 95% O₂/5% CO₂ to produce a pH of 7.2–7.4.

Electrophysiological Recording of Isolated Spinal Cord White Matter

Recording Chamber

The construction of the recording chamber is described in our previous publications [25–27]. A strip of isolated spinal cord white matter, approximately 35 mm in length, was mounted in the chamber for impulse conduction recording [28–30]. The axons were stimulated and compound action potentials were recorded at the opposite end of the strip of white matter by silver/silver-chloride wire electrodes positioned within the side chambers and the central bath. The central bath was connected to an instrument ground. Stimuli were delivered through a stimulus isolation unit and were usually in the form of 0.1 ms constant current unipolar pulses. Recordings were made using a bridge amplifier and Neurocorder (both from Neurodata Instruments). Subsequent analysis was performed using custom Labview[®] software (National Instruments[™]) on a Compaq PC[™]. Further details and description of the chamber can be found in our previous publications [26–28, 30].

Compound Action Potential Amplitude

For the recording of compound action potential (CAP) amplitude, stimuli were delivered at a frequency of one stimulus for every 3 s. A supramaximal stimulus (110% of the maximal stimulus) intensity was chosen for this test. The digitized profile of each responding CAP was recorded continuously and stored in the computer for later analysis. In addition, a real-time plot of CAP amplitude was displayed during the experiment.

Activation Threshold

Current-voltage tests were used to detect changes in activation threshold (probability) before and after ischemic insult. This test consisted of stimuli with increasing intensity in order to gradually stimulate axons of different groups with various axonal diameters to fire action potentials [31, 32]. The larger diameter axons will be activated first due to the lower threshold. The test protocol was programmed in the software and performed by the computer automatically. The stimulus intensities ranged from 0.015 to 2 mA. At each stimulus intensity level, five stimuli were repeated and an average value was used. Throughout the test, the stimulus was always delivered at a frequency of one stimulus every 3 s.

Refractory Period

The refractory period was examined by stimulating the cord with a series of twin pulses of various interstimuli intervals, ranging from 0.5 to 13 ms. A supramaximal stimulus (110% of the maximal stimulus) intensity was chosen for the refractory test. The amplitude of the first responding action potential remained the same for each pair of stimuli. The period immediately after the first stimulus when no action potential could be elicited was defined as the absolute refractory period. The time when the second responding CAP was the same height as the first one was defined as the relative refractory period. For the analysis, the amplitude of the second CAP was expressed as a percentage of the first one.

Ischemic Insult and Reperfusion with Acrolein

Once a stable CAP and membrane potential were obtained, the normal Krebs' solution was switched to a glucose-free Krebs' solution, bubbled with a 95% N₂/5% CO₂ gas mixture, to produce ischemic insult (60 min). Reperfusion was accomplished by switching back to the original, well-oxygenated Krebs' media, with or without 50 μ M acrolein. The solutions took approximately 1 min to enter the chamber, and the reperfusion was carried out for 120 min.

The Application of GSH and Ascorbic Acid

Since GSH itself is unable to cross cell membranes, glutathione ethyl ester (GEE) was utilized to gain entry into the cells due to its lipophilic nature. Spinal cord white matter strips were incubated with GEE (2 mM) during the entire period of experimentation. Two hours after the onset of GEE, the cord was subjected to a 60-min ischemic insult, followed by a 60-min 50- μ M acrolein perfusion and then a 60-min reperfusion period with normal Krebs' solution.

For the ascorbic acid study, the protocol was similar to that of GEE, except that the ascorbic acid (30 mM) was introduced to the spinal cord white matter at the same time as acrolein and remained in the perfusion until the end of the experiment. For some experiments, we altered the pH of the perfusion solution (no ascorbic acid) to equal the change in pH we obtained while using the ascorbic acid (pH 6.9). The acidic solution was then used in lieu of the ascorbic acid solution, and we followed the same protocol that was utilized during the previous study.

Horseradish Peroxidase Histochemistry

To examine the extent of anatomical damage after 60 min ischemic insult, the white matter strip in the middle chamber was removed and transferred to an oxygenated, normal Krebs' solution containing 0.015% horseradish peroxidase (HRP). After incubation for 1 h at room temperature, the tissue was fixed by immersion in 2.5% glutaraldehyde in phosphate buffer. The ventral white matter preparations were transversely sectioned at 30 μ m using a Vibratome (Electron Microscopy Science). These sections were stained with a diaminobenzidine reaction to reveal the extent of HRP uptake into damaged axons. Sections were viewed with a light microscope. Those axons labeled with HRP showed dark reaction product inside the axon [6, 26, 33]. The axons labeled with HRP were defined as unsealed axons, and those not labeled with HRP were defined as sealed axons.

Two-Dimensional Morphometry

The number of unsealed axons was counted from HRP-stained Vibratome sections. The images were first digitized and captured to a Macintosh Quadra 800 computer using a Leitz Ortoplan[®] micro-

scope and a JVC[®] video camera. Then, representative cross sections were selected from each strip, using a 6.3 \times objective. Representative area samples were also chosen from peak regions of dye uptake in transverse sections to quantify axonal sealing. The size of the region varied depending upon the thickness of the white matter. Using a 16 \times objective, axons were measured within the region. Images were first color transformed and binarized using IP Lab Spectrum. Counts of individual axons were normalized per unit area and expressed as a density (axons/mm²).

Chemicals

All chemicals, including acrolein, HRP (type IV) and GEE, were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Ascorbic acid was obtained from Mallinckrodt Co. (Paris, Ky., USA).

Statistical Treatment

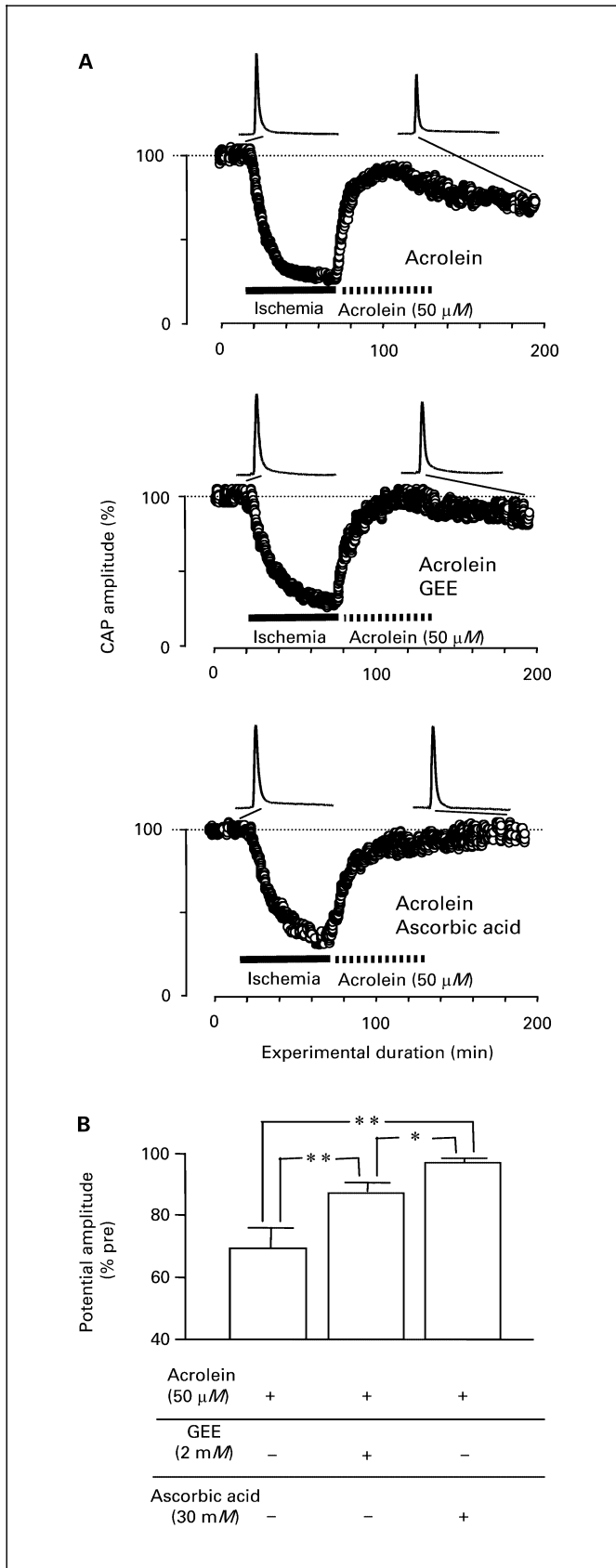
ANOVA and Tukey tests were used to compare the data related to the changes of the amplitude of CAP where multiple comparisons were made (fig. 5). Student's t test was used in all other analyses where a single comparison between two groups was made. Statistical significance was attributed to values $p < 0.05$. Averages were expressed as mean \pm standard deviation (SD).

Results

Similar to what we observed previously [6, 7], guinea pig spinal cord ventral white matter could recover almost all of its initial CAP when it was deprived of oxygen and glucose for 60 min and then reperfused with normal oxygenated Krebs' solution for an additional 120 min ($95.6 \pm 6\%$, $n = 8$). Then, in another set of experiments, following 60 min of OGD, we reperfused the cord with 50 μ M acrolein for 60 min, followed by another 60 min of reperfusion with normal Krebs' solution. The recovered CAP in these conditions was $69.1 \pm 6.6\%$ ($n = 10$) (fig. 1). In a similar experimental protocol, when GSH was introduced into the perfusion, the CAP following 120 min of reperfusion was $87.5 \pm 2.7\%$ ($n = 10$) of the initial CAP recorded before the ischemic insult. This result was significantly higher than that of the ischemia/acrolein group alone ($p < 0.001$) (fig. 1).

Similarly, when ascorbic acid was introduced into the perfusion, the CAP following 120 min of reperfusion was $97.4 \pm 1.4\%$ of the preischemic CAP ($n = 10$). This value was significantly higher than that of the ischemia/acrolein group alone ($p < 0.001$) (fig. 1) and was not significantly different than OGD alone ($p > 0.05$). However, when ascorbic acid and GSH were combined in the perfusion of the ischemic cord, the results were similar to that of ascorbic acid alone (data not shown).

In order to detect any changes of the physiological properties in the GEE or ascorbic acid-treated groups, we performed an additional series of tests designed to detect



differences that cannot be seen with our normal protocol, which was intended to monitor a single CAP in response to a constant stimulus. To detect changes in the activation threshold, we performed a current-voltage test. The test was conducted before the ischemic insult and also at the end of the reperfusion period. Figure 2 displays the activation of the preischemic condition (x-axis) plotted against postreperfusion (y-axis). The trend clearly shows that the larger diameter axons were less likely to be activated in the GEE-treated group and, to a lesser extent, the ascorbic acid-treated group. This indicates that GEE and ascorbic acid are more likely to be beneficial to smaller diameter axons than larger ones.

Next, we evaluated the changes in refractory period of the cords in the GEE or ascorbic acid-treated group. Paired stimuli with various intervals were used to elicit dual responses. In figure 3, the y-axis indicates the magnitude of the second CAP as a percentage of the magnitude of the initial CAP; the x-axis represents a log interstimulus interval ranging from 0.5 to 13 ms. Note that the postischemic plots seem to have similar absolute and relative periods compared with preischemic conditions. There was a trend for the postischemic CAP to be slightly lower than those recorded from the preischemic spinal cord white matter strips. However, these differences were not significant ($p > 0.05$).

In the ascorbic acid-treated group, we noted a change of pH following the addition of ascorbic acid into the Krebs' solution. Specifically, addition of ascorbic acid changed the pH of the Krebs' solution from 7.2 (acrolein alone) to 6.9. Therefore, it is possible that it was the pH, not the ascorbic acid, that was actually responsible for the enhanced functional recovery. In order to rule out this possibility, we adjusted the pH of the Krebs' solution to 6.9 without the addition of ascorbic acid. In this manipu-

Fig. 1. The effects of GEE and ascorbic acid on CAP recovery following ischemia/acrolein reperfusion. **A** CAP amplitude profile in the presence of GEE, ascorbic acid and acrolein alone. These graphs show the CAP recording over a period of time plotted against CAP amplitude. The values were normalized. Note the increase in CAP amplitude recovery during the reperfusion period for the two treatment groups (GEE and ascorbic acid) compared with that of acrolein alone. **B** A graphical representation of the CAP recovery with acrolein alone and the two treatment groups. With acrolein alone, there is a $69.1 \pm 6.6\%$ recovery of the CAP following an ischemic insult. The CAP recoveries of treatment with GEE and ascorbic acid were $87.5 \pm 2.7\%$ and $97.4 \pm 1.4\%$, respectively. % pre = Percentage of preischemic condition. $n = 10$ in all three groups. ** $p < 0.001$; * $p < 0.05$. ANOVA, Tukey test.

Fig. 2. Preinjury versus postinjury CAP amplitude plotted against one another at varying stimulus intensities in groups treated with GEE and ascorbic acid. Because of the fact that large caliber axons generally have a smaller threshold for activation, they are generally recruited earlier into the formation of the CAP. There is a slight tendency of the treatment groups to rescue the smaller diameter axons, which is demonstrated with the preinjury amplitude showing recruitment of around 40% of the axonal population, and at the same stimulus intensity, there was around 22% of the postinjury axonal population recruited into the formation of the CAP (GEE). A similar phenomenon, but to a lesser extent, is also displayed in the second graph (ascorbic acid). % max = Percentage of maximal stimulus.

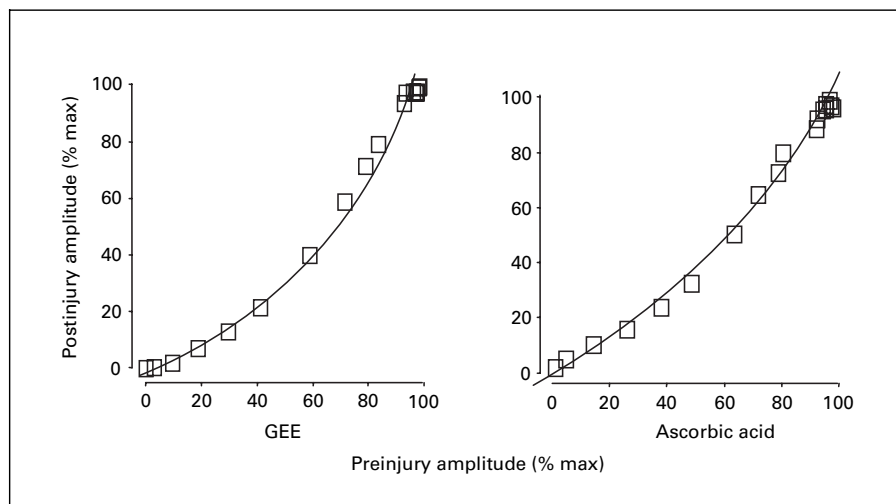


Fig. 3. Changes in the refractory period of the groups treated with GEE and ascorbic acid. These graphs show the response to a constant stimulus intensity at increasing time intervals (log scale). In this study, the posttreatment recordings had smaller percentages of CAP amplitude than the initial recordings. However, the difference is not significant.

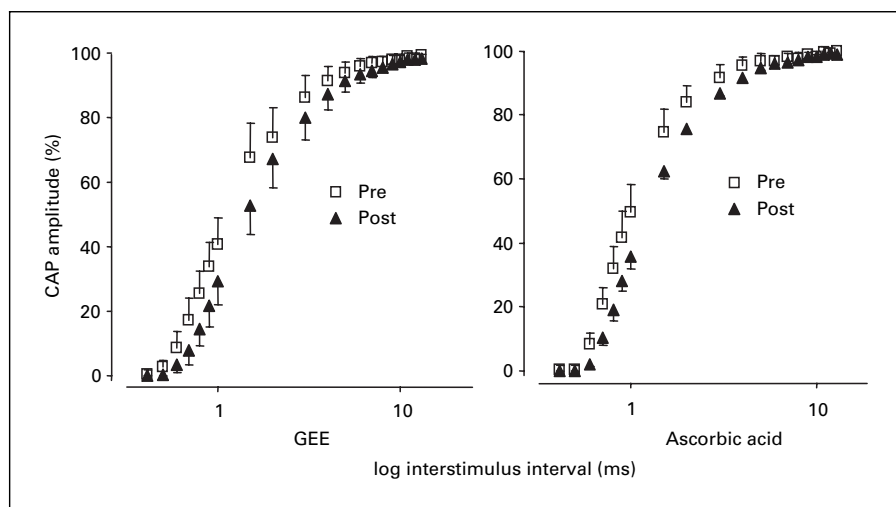


Fig. 4. The effects of pH on CAP recovery following ischemia/acrolein reperfusion. This graph demonstrates that there was a $97.4 \pm 1.4\%$ recovery of the initial CAP when the cords were reperfused with acrolein and ascorbic acid. The pH of the perfusion solution was 6.9 in this case (as compared with a pH of 7.2 with the acrolein alone). When the pH of the perfusion solution was altered to match that of the ascorbic acid perfusion (pH 6.9), the recovery ($72.6 \pm 5.1\%$) was closer to the values of the acrolein alone ($69.1 \pm 6.6\%$) rather than the treatment group, indicating that the therapeutic effects of the ascorbic acid were not due solely to alterations in the pH. % pre = Percentage of preischemic condition. $n = 10$ in all three groups. * $p > 0.05$; ** $p < 0.001$. ANOVA, Tukey test.

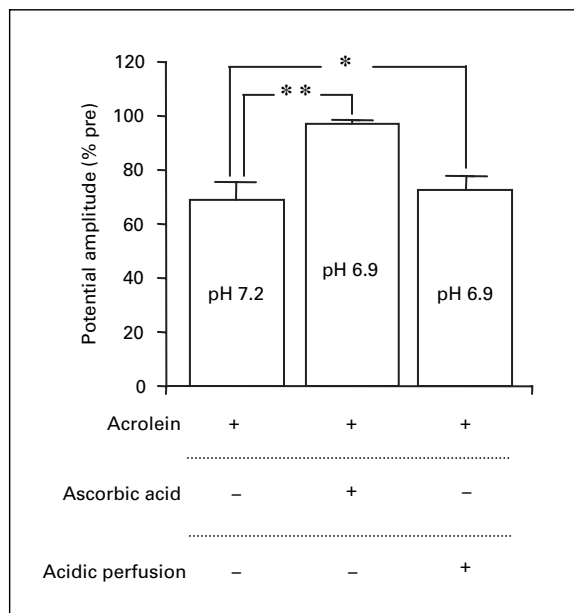
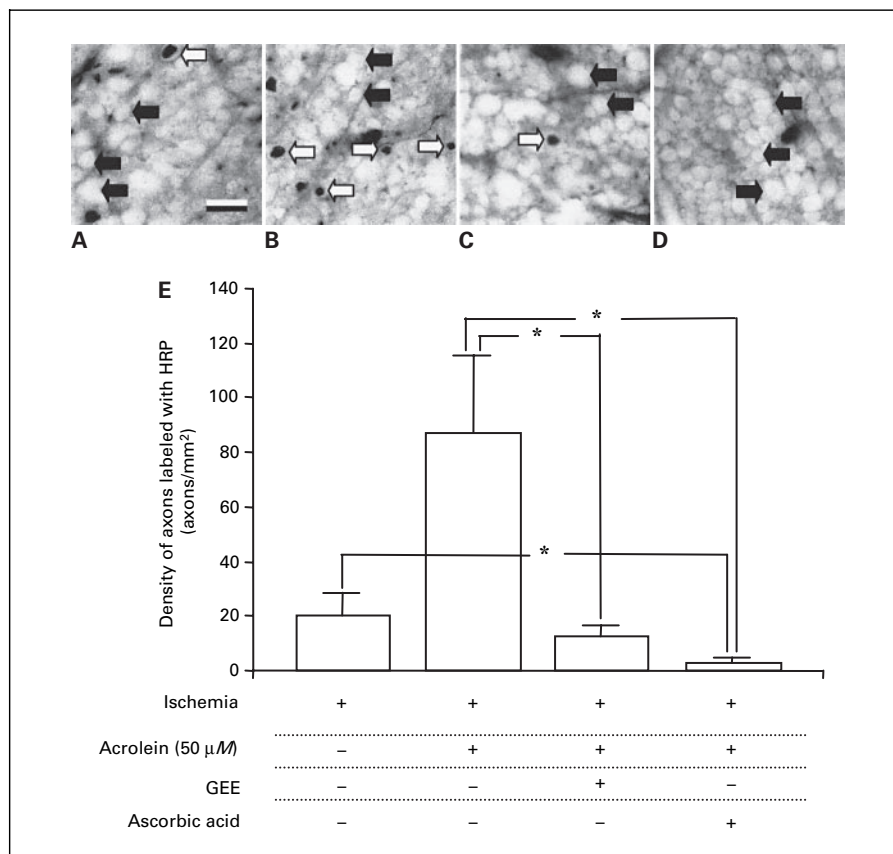


Fig. 5. Densities of axons that are labeled with HRP indicate axonal membrane disruption. The micrographs from four groups represent ischemia alone (**A**), ischemia/acrolein reperfusion (**B**), ischemia/acrolein reperfusion and GEE (**C**) and ischemia/acrolein reperfusion and ascorbic acid (**D**). White arrows indicate axons labeled with HRP; black arrows denote axons that exclude HRP. **E** The graph shows a comparison of ischemia alone, ischemia/acrolein reperfusion and the two treatment groups. Each bar represents the average axonal density (HRP labeled) \pm SD. As seen from the graph, there was a significant decrease in the amount of axons that demonstrated HRP uptake in treatment with GEE and ascorbic acid. $n = 6-10$ in all four groups. * $p < 0.001$. In addition, there is also a significant difference between the group of ischemia alone and the group of ischemia/acrolein reperfusion and ascorbic acid (* $p < 0.01$). ANOVA, Tukey test. **A-D** Scale bar = 10 μm .



lation, the recovered CAP following 120 min of reperfusion was $72.6 \pm 5.1\%$ ($n = 10$), which was not significantly different than the ischemic/acrolein group (fig. 4; $69.1 \pm 6.6\%$, $n = 10$, $p > 0.5$).

An HRP exclusion test was employed to determine whether there was any improvement in axolemmal integrity paralleling CAP conduction enhancement as a result of GEE or ascorbic acid treatment (fig. 5). In the group with 60 min of OGD and 120 min reperfusion with regular Krebs' solution, HRP labeling was 21 ± 9 axons/mm² ($n = 10$). However, when acrolein was introduced in the reperfusion period for 60 min, HRP labeling increased to 86.8 ± 29.1 axons/mm² ($n = 6$), which was significantly higher than OGD alone ($p < 0.01$). In the GEE treatment group, the HRP-labeled axon density was 12.32 ± 4.3 axons/mm² ($n = 6$), which was significantly lower than acrolein-reperused cords without treatment ($p < 0.001$). To a greater extent, treatment with ascorbic acid reduced HRP labeling to 2.94 ± 1.8 axons/mm² ($n = 6$, $p < 0.001$) compared with the ischemic/acrolein group. In addition, HRP labeling in the ascorbic acid-treated group is significantly lower than that in the group of OGD alone ($p < 0.001$).

Discussion

Using this *in vitro* OGD model, we have shown that spinal cord ventral white matter can recover nearly all of its action potential conduction following a 60-min OGD and a 120-min reperfusion with regular Krebs' solution containing normal oxygen and glucose (current study) [6, 7]. In addition, we have also shown that 50 μM acrolein, an LPO by-product that is significantly increased following traumatic spinal cord injury [8], did not cause any noticeable damage when exposed to otherwise uninjured isolated white matter axons [9]. However, when acrolein (50 μM) was introduced during the reperfusion period, an irreversible decrease of CAP conduction resulted (current study) [7]. These results lead to the hypothesis that acrolein and ROS may play an important role in ischemic injury. We have further hypothesized that acrolein-mediated injury was induced mainly through the stimulation of ROS elevation [10]. The data from this study is consistent with such hypothesis by demonstrating that GSH and ascorbic acid, two effective ROS scavengers, are independently capable of countering acrolein-mediated functional loss and anatomic deficits.

The form of GSH we used in this study was GEE. Unlike GSH, GEE is membrane permeable and capable of crossing cell membrane [16]. Once inside the cell, cytosolic esterase will cleave the ester group and activate the GSH [16]. GSH is then capable of scavenging ROS and, more importantly, binding directly to acrolein [34, 35]. The fact that GSH can significantly protect the axons from ischemia/acrolein-mediated damage indicates that GEE did indeed diffuse into the spinal cord tissue and enter the cell at most of the locations. This demonstrates that the surface perfusion of GEE is an effective method to deliver GSH, which is of importance in the potential use of this compound in spinal cord injury victims.

It is known that GSH can directly bind to and detoxify acrolein, which may also contribute to the overall effect of GEE in reducing acrolein-mediated damage as seen in this study [34, 35]. Pocerlich et al. [35] have also shown that GSH elevation alleviates acrolein-induced tissue damage in an *in vitro* preparation. Lucas et al. [19] have further shown that GSH augmentation can significantly suppress LPO after an *in vivo* spinal cord injury. Despite the compelling evidence of suppressing free radical injury and LPO, the effect of GSH in reducing spinal cord tissue damage and enhancing neurological function is still controversial [20, 36]. Our study certainly suggests that GSH is capable of enhancing functional recovery in spinal cord tissues against ischemic and oxidative insults.

Unlike GSH, ascorbic acid is not known to bind acrolein directly [16]. Therefore, ROS scavenging will be the major effect of ascorbic-mediated protection against ischemia/acrolein-mediated damage. It is clear that ascorbic acid can effectively restore CAP conduction and membrane integrity (fig. 1, 4). This suggests that ROS mediates most, if not all, of ischemia/acrolein-induced functional and structural damage in spinal cord white matter. In addition to the protective effects in spinal cord white matter, similar protection of ascorbic acid against acrolein-induced damage was demonstrated in cultured human bronchial epithelial cells [37]. This further stresses the role of ROS in acrolein-mediated damage in nervous tissue as well as other tissues. It is interesting to note that in the ischemia/acrolein reperfusion and ascorbic acid-treated group, the HRP labeling is even lower than in the group of ischemia alone. This suggests that a low level of ROS existed in the group of ischemia alone, which inflicted low levels of membrane damage denoted by HRP labeling, which is probably negligible compared with overall axonal population.

The differential efficacy of GEE and ascorbic acid in inhibiting functional and anatomical damage noticed in

the current study may be partially explained by the fact that central nervous system tissue can take up ascorbic acid significantly better than GSH [38]. The other possibility is simply that the ascorbic acid concentration used in the current study is much higher than GSH. A complete dose-dependent curve, as well as a measurement of correspondent tissue levels of GSH and ascorbic acid, would clarify this issue.

In our previous studies, we have confirmed that there is no differential vulnerability of small or larger axons in either OGD alone or OGD/acrolein-treated spinal cord white matter [6, 7]. However, in this study, we have noted that GEE, and to a lesser extent ascorbic acid, have a tendency to rescue smaller rather than larger diameter axons (fig. 2). The mechanism of this phenomenon is not clear. It has been shown that the small axons are relatively less vulnerable to metabolic deficits or ATP depletion [6]. Likewise, the small axons may be more responsive to antioxidant treatment, which likely enhances energy production from mitochondria as a result of ROS scavenging. We speculate that, unlike the studies using sodium cyanide and sodium azide [6], reduction of residual ATP may not be the major deficit in OGD/acrolein-mediated damage. This may explain why little differential damage between small and large axons was observed in a combined injury [7]. However, in GEE- and ascorbic acid-treated groups, the increase in ATP may be a dominant factor in enhancing functional recovery. Since larger axons are likely to be metabolically more demanding, the limited increase of ATP may be more influential in smaller axons than larger axons. Whatever the mechanism is, the differential benefit between smaller and larger axons may have a profound impact on the prediction of the type of functional recovery upon GSH and ascorbic acid treatment of spinal cord injury.

Even though GEE and ascorbic acid significantly restore impulse conduction and membrane integrity, there is a concomitant, slight tendency of increased refractoriness. Specifically, during dual stimuli responses, there was a trend for the postischemic CAP to be slightly lower than those recorded from the preischemic CAP, although these differences were not significant (fig. 3; $p > 0.05$). It is likely that the newly recruited axons are responsible for this change, compared with the healthier axons that could conduct action potentials before the application of GSH and ascorbic acid. The simplest explanation would be that although some axons are able to conduct in the presence of GSH or ascorbic acid, the safety factor for conduction remains significantly compromised by damage to paranodal structures and the increased capacitive load provided by the internodal membrane [39, 40].

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