Therapeutic hypothermia effectively reduces elevated extracellular ascorbate concentrations caused by acute spinal cord injury

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Introduction

Traumatic spinal cord injury (TSCI) is a disaster affecting approximately 15 to 40 people per million every year [1]. It is one of the most serious diseases in clinics, which can be caused by trauma ranging from traffic accidents to war wounds [2]. The damage related to SCI is sorted in two phases: primary and secondary. The primary injury is immediate and irreversible; the secondary injury is caused by numerous chemicals, including free radical and excitatory amino acids, which are thought to be responsible for both motor and sensorial defects after the primary impact [3]. Besides that, the long-term functional and histological damage after spinal cord injury could also be caused by a series of acute neurochemical imbalance inducing pathological changes such as excessive oxidative stress [4] and glutamate neurotoxicity [5, 6].

As one kind of important biochemical species, ascorbate is known to be involved in many neurochemical processes. It is one of the most significant antioxidants and free radical scavengers that relieve oxidative stress in the central nervous system (CNS) [7]. In addition, ascorbate also acts as a neuromodulator in glutamate-mediated neurotransmission in the central nervous system [8]. Compared with other tissues, the high concentration of ascorbate in the nerve tissue also strongly suggests that ascorbate plays a very important role in neurophysiological and pathological processes [9]. In comparison to the many studies done on the extracellular ascorbate concentration and roles in the brain, concentration in the spinal cord has been less studied. Preliminary study of ascorbate reported that spinal cord injury induces an increase of extracellular ascorbate concentration in anesthetized rats [10]. However, ascorbate changes during hypothermic intervention remain to be fully understood. For the past few years, systemic hypothermia has taken the spotlight for its use in SCI research fields. In clinical trials, especially in the treatment of acute spinal cord injury, cardiac arrest and neonatal asphyxia, therapeutic systemic hypothermia can be achieved by surface cooling using ice blankets, cooling packs or soaking ice bath. Intravascular cooling catheters and body cavity lavage can achieve faster systemic cooling [11]. Numerous clinical studies have shown that therapeutic hypothermia used in patients with severe acute spinal cord injury dose not significantly increase the risk of complications [12, 13]. The clinical case of a national football league athlete has recently caused widespread concern. He suffered a severe cervical spinal cord injury in 2007 and now has achieved good prognosis through early application of therapeutic hypothermia [14]. Clinic evidence for the application of therapeutic hypothermia in patients with severe spinal cord injury is encouraging. Studies demonstrated that hypothermia reduces secondary pathological mechanisms, such as inflammation, excitotoxicity, oxidative stress, edema, and apoptosis, but detailed molecular mechanisms are still not fully understood. The results above intensively indicate that ascorbate is...
involved in several pathological processes during the acute periods after SCI and therapeutic hypothermia administration. However, as we know, detailed neurochemical mechanisms have not been explored yet. Thus, exploring ascorbate dynamics and molecular mechanism underlying spinal cord injury is worthwhile, especially under therapeutic hypothermia administration.

In this article, we, for the first time, demonstrate the dynamic changes of ascorbate in the acute phases of spinal cord injury and therapeutic hypothermia interference with a reliable electrochemical platform that can be used for continuously monitoring ascorbate of the spinal cord at different states. By taking advantage of the electro-analytic activity of single-walled carbon nanotubes (SWNTs) to electrochemically oxidize ascorbate, the system has proven to be very suitable for studying the changes of ascorbate concentration independent of O₂ and pH fluctuation and other coexisting interference in the spinal cord. We find that hypothermia therapy can effectively reduce the acute increase of extracellular ascorbate concentration after acute spinal cord injury, which provides necessary experimental information for the later development of related treatment.

**Materials and methods**

**Animals and study groups**

Adult male Sprague-Dawley rats (3 months of age, weighing 300 ± 50 g at the time of surgery) were housed individually under 12 h light/dark cycle and had food and water *ad libitum* at room temperature (22 ± 3 °C). Each rat was tested and normal motor function was found before surgery.

To study the spinal cord response induced by SCI and the following therapeutic hypothermia treatment, rats were randomly divided into four experimental groups: A (normothermia control group: laminectomy + normothermia treated), B (hypothermia control group: laminectomy + hypothermia treated). To demonstrate whether therapeutic hypothermia or Long-term monitoring will change the ascorbate basal level of spinal cord without injury. C (normothermia group: laminectomy + spinal cord injury + normothermia treated); D (hypothermia group: laminectomy + spinal cord injury + hypothermia treated). Each of group contained five rats.

**Surgical technique**

The animals were anaesthetized with urethane (10% w/v; 8 mL kg⁻¹ ip.) and were placed on the self-developed temperature-changeable aluminum plate with the water circulation heating/cooling system. A midline dorsal incision was performed using sterile technique. The T8-L2 laminae were exposed by dissecting the paravertebral muscles. Laminctomy was performed at T9-T12 levels. SCI was accomplished by extradural compression of the exposed spinal cords at T10 level using an aneurysm clip (Yasargil FE760, force of closure 70 g) in each case, as described by Murat Kalayci [15].

**Control of rat body temperature**

To maintain and control the systemic temperature in the rats, a self-developed temperature-changeable aluminum plate with water circulation was used. The rectal temperature of the rat was continuously measured with a precision thermometer connected to the water heating/cooling device. When the body temperature of the rat exceeds or falls below the set temperature, the device automatically cools or heats. During the entire experiment, except for the animals treated with hypothermia, the rats’ temperature was maintained at 37 ± 1 °C as measured by rectal probe and the plate temperature was set to 37 °C. When animals were treated with hypothermia, the plate temperature was set to 28 °C. The target temperature of 28 ± 1 °C was reached within 15 min and then maintained.

**Hematoxylin–eosin (HE)staining**

The 5-μm tissue sections that were made from paraffin-embedded blocks of the spinal cord specimens fixed by formalin, extracted from the injured animals from Group C and Group D, were subjected to routine hematoxylin and eosin staining and evaluated by microscope.

**In vivo micro-dialysis and online electrochemical system for ascorbate**

In vivo microdialysis was performed by implanting a microdialysis probe (2 mm in length; Bioanalytical Systems Inc. (BAS), BAS Carnegie Medicine) into the dorsal horn at T-11 after laminectomy. One probe corresponds to one rat. After equilibrating for 90 min by continuously perfusing with artificial cerebrospinal fluid (aCSF) (126 mmol L⁻¹ NaCl, 2.4 mmol L⁻¹ KCl, 1.1 mmol L⁻¹ CaCl₂, 0.85 mmol L⁻¹ MgCl₂, 27.5 mmol L⁻¹ NaHCO₃, 0.5 mmol L⁻¹ Na₂SO₄, 0.5 mmol L⁻¹ KH₂PO₄, pH 7.0) at a flow rate of 3 μL min⁻¹ driven by a micro injection pump (CMA/100; CMA Micro-dialysis AB, Stockholm, Sweden), the spinal cord microdialysate was directly delivered into a thin-layer radial electrochemical flow cell through tetrafluoroethylene hexafluoropropene (FEP) tubing for continuously monitoring ascorbate. In the first 20 min, the animals were kept at normal body temperature to determine the basal level. Then, according to the grouping conditions, continue to maintain normothermia or hypothermia treatment. The online electrochemical method used for continuously monitoring spinal cord consists of selective electrochemical detection with a thin-layer electrochemical flow cell (BAS) and in vivo micro-dialysis. The thin-layer electrochemical flow cell is made up of a thin layer radial flow block equipped with a glassy carbon (GC) electrode (6-mm diameter) as the working electrode, an Ag/AgCl electrode (3 mol L⁻¹ NaCl) as the reference electrode and a stainless steel as counter electrode. The thickness of the gasket used was 50 mm. To achieve the specificity for ascorbate measurements, the GC electrode was modified with the heat-treated single-walled carbon nanotubes (SWNTs) with a method reported in our earlier work [16]. Briefly, GC electrodes were polished first with emery paper and then with aqueous...
slurries of fine alumina powders (0.3 and 0.05 mm) on a polishing cloth. The electrodes were finally rinsed with doubly distilled water under an ultrasonic bath for 10 min. A 2 mg mL\(^{-1}\) of heat-treated SWNTs were dispersed into N,N-dimethyl formamide and 4 mL of the dispersion was coated onto GC electrodes. The SWNT-modified GC electrodes were air-dried to evaporate the solvent before use. The experimental setup for continuous online measurements of extracellular ascorbate by integrating electrochemical detection with in vivo micro-dialysis was schematically shown in Scheme 1.

Statistics

For statistical analysis of the microdialysate ascorbate levels, according to the linear calibration equation introduced later, the current responses recorded with our OECS were converted into the concentration (\(\mu\)mol L\(^{-1}\)) of ascorbate. The levels of microdialysate ascorbate were reported as the percentage of their basal level. The data were reported as the mean ± standard deviation (SD). Ascorbate levels at the time point of 80 min after the start of measurement were compared with their basal level by paired \(t\) test. \(p < .05\) was considered as significantly different.

Results

Online electrochemical system (OECS)

The online electrochemical method shows a linear response toward ascorbate, as shown in Figure 1, within the concentration range from 1 \(\mu\)mol L\(^{-1}\) to 100 \(\mu\)mol L\(^{-1}\) (I (nA) = 6.95 CAA(\(\mu\)mol L\(^{-1}\)) + 9.51) with a linear coefficient of 0.999. The detection limit was calculated to be 0.20 \(\mu\)mol L\(^{-1}\) based on a signal-to-noise ratio of 3. Moreover, this electrochemical method which integrates online electrochemical detection with in vivo micro-dialysis was found to be very stable for continuous monitoring of extracellular ascorbate.

OECS for spinal cord ascorbate detection

To verify that the OECS could be used for monitoring ascorbate of hypothermia in SCI, the change of ascorbate in the spinal cord in the sham-operated rats induced by hypothermia or Long-term monitoring need to be studied first. For this purpose, we continuously monitored the spinal cord ascorbate dynamics in Group A and Group B with our OECS method. Figure 2 depicts typical current-time responses, and the statistic results of ascorbate change in the spinal cord in the control group. As typically shown in Figure 2(A), basal ascorbate concentration of spinal cord without injury did not change during long-term monitoring process (upper trace). The statistical analysis, shown in Figure 2(B) (upper trace), suggests the ascorbate concentration at the 80 min after beginning the recording has no significant difference compared with the basal ascorbate level (paired \(t\) test, \(n = 5\)).

Similar with the results obtained with the Group A, hypothermia does not induce any obvious change of extracellular ascorbate level in the spinal cord within 80 min measurement (paired \(t\) test, \(n = 5\)) in the Group B rats, as displayed in Figure 2(A,B) (lower trace).

These results demonstrate that extracellular ascorbate level of the spinal cord remains constant during either long-term monitoring process or hypothermia process. These features substantially provide the basis of our OECS coupled with in vivo micro-dialysis for real-time ascorbate detection for assessing the neuroprotective efficiency of hypothermia through continuously monitoring spinal cord ascorbate in SCI rat, as demonstrated later.

Spinal cord ascorbate change after SCI and hypothermia

With the OECS, we studied ascorbate dynamics of the injured spinal cord which was treated with normothermia or hypothermia. As displayed in Figure 3(A) (upper trace) and Figure 3(B) (upper trace), after SCI the spinal ascorbate increases...
immediately and then decreases slowly, finally reaches $2.36 \pm 0.65 \mu\text{mol L}^{-1}$ with little change ($164.90\% \pm 7.99\%$ of the basal level) at the time point of 60 min after SCI (paired $t$ test, $p < 0.05$, $n = 5$). To our surprise, with hypothermia administration immediately after SCI, the ascorbate level was $3.01 \pm 0.59 \mu\text{mol L}^{-1}$ ($100.24\% \pm 5.02\%$ of the basal level) at the time point of 60 min after SCI, as shown in Figure 3(A,B) (lower trace). The statistical analysis also suggests the
Ascorbate concentration at the 60 min after SCI has no significant difference compared with the basal ascorbate level (paired t test, n = 5). Although the previous studies have suggested that hypothermia treatment after SCI could change glutamate concentration in subacute and chronic phase after SCI, as we know, it is the first time we observed the effects of hypothermia treatment on the SCI-induced ascorbate change in the rat spinal cord.

Hypothermia reduce SCI-associated damage

The HE staining data of transverse spinal cord sections and the data of local position around the implanted part of the probe were shown to determine the contribution of hypothermia to the spinal cord repair (Figure 4). Tissue degradation of white and grey matter appeared in both Group C and Group D. Major spinal cord tissue destruction occurs at and near the center of the lesion. Significant traumatic manifestations were observed with Group C, including widespread vacuolation, loosened structure, hemorrhage, obvious edema. Compared with Group C, the traumatic manifestations were obviously reduced in Group D, as demonstrated by less vacuolation, a rough normalization of the tissue structure and less edema. These results showed that hypothermia could effectively mitigate SCI-associated damage in spinal cord.

Discussion

This is the first report concerning effect of hypothermia on extracellular ascorbate in SCI with OECS. The most important finding is that therapeutic hypothermia effectively reduces elevated extracellular ascorbate concentrations caused by acute spinal cord injury. The specificity study shown in Figure 1 demonstrates that online electrochemical method has a good selectivity and linear response for ascorbate. These features substantially enable it well competent for effectively monitoring of extracellular ascorbate during the acute phase of spinal cord injury. It is worth noting that although high-performance liquid chromatography (HPLC) combined with off-line electrochemical detection can be used to measure ascorbate [10, 17], our detection platform remains prominent due to the less technical requirements feature and chemical instability of ascorbate, avoiding sample collection, and more importantly, the near real-time feature of the OECS. The in vivo HPLC method run the chromatography barely every ten min, which makes it not a real-time method for the SCI model considering the components may change every minute. The off-line HPLC methods used for detecting spinal cord ascorbate might be time consuming and lacks temporal resolution. In addition, the procedure of HPLC may cause the oxidation of ascorbate in atmosphere, which leads wrong value from the real one. As shown in our earlier research [16, 18, 19], our OECS coupled with in vivo
micro-dialysis has high specificity, good accuracy, and stability for monitoring ascorbate continuously. More notably, this system is highly resistant to pH and O₂ fluctuation under pathological conditions.

As depicted in Figure 2, the current response recorded for the spinal cord microdialysate continuously sampled from the spinal cord almost remained constant for at least 1 h, indicating that our system can achieve long-term stable measurement. This is understandable because the extracellular ascorbate dynamically self-regulates through homeostasis. For instance, excess ascorbate can be transported into neural cells via sodium-dependent vitamin C transporter 2 (SVCT2) [20]. And we found no significant difference in the ascorbate concentrations between normothermic and hypothermic animals without injury at the end of the experiment, indicating that there was no rate-limiting difference in diffusion rate across the micro-dialysis membrane during hypothermia. These features substantially provide the basis of our method to detect ascorbate for assessing the neuroprotective efficiency of hypothermia in vivo through real-time monitoring spinal cord ascorbate of SCI rats with normothermia or hypothermia treated.

As shown in Figure 3, when the spinal cord injury occurs, extracellular ascorbate concentration in the injured area increases sharply. This may be due to the inactivation of the channel protein or the destruction of integrity of the cell membrane after the death of the nerve cell [21]. The high concentration of ascorbate stored in the cell will be instantly released in large quantities resulting in a significant increase in extracellular ascorbate concentration. The increase in ascorbate, immediately following injury could increase the overall reducing ability of the extracellular fluid and provide certain protection against oxidative damage of outer cell membranes [22]. However, the ascorbate concentration still reached a stable value at about 60 min after injury which was approximately twice as much as the basal level of the spinal cord although it slowly decreased for 0.5 h. The mechanisms of this increase might be due to the coupling of glutamate uptake to ascorbate release via a hetero-exchange transporter. Ischemia following spinal cord injury is its basic pathological change [23]. Ischemia causes a reduction and even disappearance in oxygen and sugar supply, decreasing ATP production and energy supplement. In the process of energy depletion, the reduction of ATP decreased the activity of Na⁺/K⁺-ATPase, and the glutamate transport was stopped or reversed. At the same time, after the depolarization of neurons, the synaptic release of glutamate caused an increase in extracellular glutamate concentration. The decrease in ATP also blocks the ATP-dependent glutaminase synthases, interrupts the glutamate-glutamine cycle, causes intracellular glutamate to accumulate, and when it reaches a certain concentration, it transcends the ability to transport glutamate into the cell. The concentration of extra glutamate increased [24]. Glutamates release and accumulate during SCI, resulting in neurotoxicity through the direct damage of neurons and oligodendrocytes, and also through the production of reactive oxygen species. Thus, ascorbate release induced by lesion may facilitate the uptake of glutamate accumulated in the extracellular fluids to avoid its accumulation. Glutamate-ascorbate hetero-exchange mechanism explains the phenomenon that glutamate uptake by nerve cells could facilitate ascorbate efflux [25]. Astrocytes [26] and SH-SYSY neuroblastoma cells [27] also conform to this effect. In addition to the above mechanisms, cell edema caused by injury activates Volume-sensitive osmolyte and anion channels (VSOAC) of nerve cells also contribute to the increase. Volume-sensitive osmolyte and anion channels (VSOAC) – also known as volume-regulated anion channels (VRAC) – are

Figure 4. Effects of hypothermia on the histological changes in the spinal cord in rats with spinal cord injury. The spinal cord tissue harvested at 1h after the spinal cord injury was subject to hematoxylin-eosin (HE) staining and was examined by microscope for the identification of tissue damage (n = 6). The results presented in the text are representative.
functionally defined plasma membrane anion channels involved in regulating cell volume, which were first thought to play a role in ascorbate efflux in cultured astrocytes under conditions simulating brain edema [28]. In fact, changes in cell volume are induced by elevated intracellular osmotic pressure, as discovered under some pathological conditions such as hypoxia, ischemia or metabolic disorders [29]. VSOACs, activated by cell swelling, play an important role after in the so-called cell regulatory volume decrease (RVD) [30]. VSOACs can facilitate the efflux of organic osmoles such as polyols and amino acids induced by hypo tonicity. Interestingly, they can also efflux ascorbate [31]. There are also reports in the literature that the Ca\(^{2+}\) influx also triggers the release of ascorbate [32].

To our knowledge, the attenuation of the SCI-induced spinal cord ascorbate increase caused by immediate hypothermia treatment in such a short time after injury has not been reported so far. Immediately after spinal cord injury was treated with therapeutic hypothermia, the magnitude of ascorbate decreased more significantly than in the injury group as shown in Figure 3. Most scholars believe that therapeutic hypothermia can significantly reduce the excessive release of excitatory amino acids after central nerve injury. Research has shown that hypothermia plays a protective role by down-regulating the reverse transport of GLT-1 to reduce the levels of extracellular glutamate induced by injury [33]. The treatment of therapeutic hypothermia immediately after SCI reduces oxygen consumption and delays the onset of post-injury energy failure [34]. The effect of reducing oxygen metabolism may also contribute to reduced excitatory amino acid release [11]. The decrease of glutamate release reduced the hetero-exchange of ascorbate with extracellular glutamate, and the reduction of intracellular ascorbate efflux, achieving a significant decrease in the ascorbate concentration in the group D versus the group C. In addition, mild hypothermia significantly inhibits Ca\(^{2+}\) influx during hypoxia [35], effectively protects the integrity of the central nervous cell membrane during acute injury, and reduces the sustained massive outflow of intracellular ascorbate and calcium-dependent ascorbate efflux [36]. As the HE staining data shows, Group D is associated with less histologic evidence of spinal cord edema, and the less cell edema caused less activates VSOACs of nerve cells which also contribute to that extracellular ascorbate concentration returns to basal level at the time point of 60 min after SCI.

**Conclusion**

In summary, our study provides direct experimental evidence indicating hypothermia therapy can effectively reduce the acute increase in extracellular ascorbate concentration after acute spinal cord injury. Preliminary conclusions are drawn that a significant reduction in spinal cord ascorbate concentration in rats with spinal cord injury under mild hypothermia may be related to protective mechanisms associated with secondary spinal cord injury. Therefore, hypothermia might serve as a treatment to reduce secondary spinal cord injury and promote spinal cord repair. With the in-depth study of mild hypothermia, it is believed that in the near future, the clinical application of hypothermia will gradually mature.

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