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Original Contribution

Therapeutic treatment with ascorbate rescues mice from heat stroke-induced death by attenuating systemic inflammatory response and hypothalamic neuronal damage

Chia-Yu Chang [a,b,c], Jen-Yin Chen [d], Sheng-Hsien Chen [e], Tain-Junn Cheng [a,f], Mao-Tsun Lin [g], Miao-Lin Hu [b,*]

[a] Department of Neurology, Chi Mei Medical Center, 901 Chung-Hwa Road, Yong kang, Tainan, Taiwan
[b] Department of Food Science and Applied Biotechnology, National Chung-Hsing University, 250 Kuo-Kuang Road, Taichung 402, Taiwan
[c] Center for General Education, Southern Taiwan University of Science and Technology, 1 Nan-Tai Street, Yong kang, Tainan, Taiwan
[d] Department of Anesthesiology, Chi Mei Medical Center, Tainan, Taiwan
[e] Da-An Women and Children Hospital, Tainan, Taiwan
[f] Department of the Senior Citizen Service Management, Chia Nan University of Pharmacy and Science, Tainan, Taiwan
[g] Department of Medical Research, Chi Mei Medical Center, Tainan, Taiwan

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ABSTRACT

The impact of ascorbate on oxidative stress-related diseases is moderate because of its limited oral bioavailability and rapid clearance. However, recent evidence of the clinical benefit of parenteral vitamin C administration has emerged, especially in critical care. Heatstroke is defined as a form of excessive hyperthermia associated with a systemic inflammatory response that results in multiple organ dysfunctions in which central nervous system disorders such as delirium, convulsions, and coma are predominant. The thermoregulatory, immune, coagulation and tissue injury responses of heatstroke closely resemble those observed during sepsis and are likely mediated by similar cellular mechanisms. This study was performed by using the characteristic high lethality rate and sepsis-mimic systemic inflammatory response of a murine model of heat stroke to test our hypothesis that supra-physiological doses of ascorbate may have therapeutic use in critical care. We demonstrated that parenteral administration of ascorbate abrogated the lethality and thermoregulatory dysfunction in murine model of heat stroke by attenuating heat stroke-induced accelerated systemic inflammatory, coagulation responses and the resultant multiple organ injury, especially in hypothalamus. Overall, our findings support the hypothesis and notion that supra-physiological doses of ascorbate may have therapeutic use in critical care.

1. Introduction

Vitamin C (ascorbic acid) dissociates at physiological pH to form ascorbate. It is well known that ascorbate acts physiologically as an antioxidant and co-factor in the biosynthesis of many neurotransmitter sand neuropeptides [1]. The impact of ascorbate on oxidative stress-related diseases is moderate because of its limited oral bioavailability and rapid clearance. However, recent evidence of the clinical benefit of parenteral vitamin C administration has emerged, especially in critical care [2]. Supra-physiological doses of ascorbate by parenteral administration were recently shown to exert inflammation inhibitory and organ-protective effects in several critical conditions, such as sepsis, cardiac arrest and burn injury [3]. In animal models of sepsis, intravenous ascorbate increases survival and protects several microvascular functions, microvascular permeability barrier, and arteriolar responsiveness to vasoconstrictors and vasodilators [4,5]. The effects of parenteral ascorbate on microvascular function are both rapid and persistent. It is suggested that ascorbate quickly accumulates in microvascular endothelial cells, which scavenges reactive oxygen species (ROS) and modulates redox-sensitive signaling pathways to diminish septic induction of NADPH oxidase and inducible nitric oxide synthase [6].

Heatstroke (HS) is defined as a form of excessive hyperthermia associated with a systemic inflammatory response that results in multiple organ dysfunctions in which central nervous system disorders such as delirium, convulsions, and coma are predominant [7]. HS-induced deaths are increasing with global warming and with a world-wide increase in the frequency and intensity of heat waves [8]. The thermoregulatory, immune,

* Corresponding author.
E-mail address: mlhuhu@nchu.edu.tw (M.-L. Hu).

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coagulation and tissue injury responses that ensue during long-term progression of heatstroke closely resemble those observed during sepsis and are likely mediated by similar cellular mechanisms [9]. In addition, it is suggested that ischemic and oxidative damage to the hypothalamus may be responsible for HS [10]. Hence, we focused on measuring HS-induced neuronal cell damage in the hypothalamus for its pivotal role in thermoregulation and control of autonomic and endocrine activity [11].

In this study, by using the characteristic high lethality rate and sepsis-mimic systemic inflammatory response of a murine model of HS, we tested our hypothesis that supra-physiological doses of ascorbate may have therapeutic use in critical conditions, HS as an exemplar. We measured the lethality rate, systemic inflammatory (serum levels of TNF-α and IL-10), coagulation (tissue factor) and multiple tissue injury (iNOS, cell apoptosis) responses in lung, kidney and hypothalamus (the thermoregulatory center) tissues in HS mice, and we evaluated whether ascorbate administration can attenuate these parameters.

2. Materials and methods

2.1. Murine model of heatstroke

All the experiments were carried out in accordance with the ethical guidelines laid down by the committee for the purpose of control and supervision of experiments on animals, Chi Mei Medical Center, Tainan, Taiwan (IACUC no: 101122426). Institute of Cancer Research (ICR) inbred male mice were given food and water ad libitum and acclimatized to room temperature at 22 ± 2 °C relative humidity of $50 \pm 8\%$, and a 12-h dark/light cycle for 1 week before the start of the experiment at least. ICR male mice 8 to 10 weeks old were exposed to whole-body heating (WBH) (41.2 °C relative humidity 50–55%, and for 1 h) in an environment-controlled chamber [12]. The time at which mice were removed from the environmental chamber was designated as 0 h. The WBH-treated mice were returned to room temperature (24 °C) at the end of WBH. Mice that survived on day 7 of WBH were considered survivors, and the data were used for analysis of the results. Core temperatures were measured every 4 h for period of 24 h with a copper constantan thermocouple inserted into the rectum and connected to a thermometer (HR1300; Yokogawa, Tokyo, Japan). In separate experiments, 4 h after WBH, all animals were killed and their organs were removed for histology, serum enzyme linked immunoabsorbent and immunohistochemistry assays. After the 1-h heating period, animals were properly fed and hydrated. Heat stroke resembles sepsis in many aspects [7]. As in many sepsis studies, we used death as an end point in conscious mice in this study.

2.2. Experimental groups

Animals were assigned randomly to one of three groups: (1) the normothermic control (NC) mice, which were exposed to room temperature (24 °C) throughout the entire experiments. (2) heatstroke (HS) mice treated with saline (HS+saline), in which saline was injected intraperitoneally (1 mL/kg, i.p.) immediately after the end of WBH, and (3) heat stroke (HS) mice treated with ascorbate (HS+ascorbate) in which ascorbate were injected intraperitoneally (500 mg/kg, i.p.) immediately after the end of WBH. Ascorbate was dissolved in saline (1 ml/kg) with an injection volume equal to that of saline vehicle. Before the start of experiments, their core temperature was within the normal body temperature range of 37.0–37.6 °C.

2.3. Assessment of thermoregulatory function

Immediately after the termination of WBH, the animals were returned to a room temperature of 24 °C for recovery. According to the findings of Chatterjee et al. [12], WBH-treated mice became hypothermia, when they were exposed to room temperature (24 °C).

2.4. Neuronal damage score

At the end of the experiments, animals were killed by an overdose of sodium pentobarbital, and the brains were fixed in situ and left in skull in 100% neutral-buffered formalin for at least 24 h before removal from the skull. The brain was removed and embedded in paraffin blocks. Serial sections (4 μm thick) through the hypothalamus were stained with hematoxylin and eosin for microscopic examination. The extent of neuronal damage was scored on a scale of 1–3, modified from the grading system of Pulsinelli et al. [13], in which 0 is normal, 1 indicates that approximately 30% of the neurons are damaged, 2 indicates that approximately 60% of the neurons are damaged, and 3 indicates that 100% of the neurons are damaged. Each hemisphere was evaluated independently by an examiner blinded to the experimental conditions.

2.5. Serum Tumor Necrosis Factor (TNF) –α and interleukin (IL)-10 assays

Blood specimens at 4 h after termination of WBH were drawn by heart puncture from the mice after they had been anesthetized with urethane (1.4 g/kg body weight; i.p.). The blood was centrifuged to isolate the upper layer of serum. Serum concentrations of TNF-α and IL-10 were determined using double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction. The concentration of TNF-α, or IL-10 in the serum samples was calculated from the standard curve multiplied by the dilution factor and was expressed as picograms per milliliter.

2.6. Terminal Deoxy-nucleotidyl Transferase-mediated dUTP Nick end Labeling (TUNEL) assay

The HS mice were given an overdose of general anesthesia with urethane (1.4 g/kg body weight; i.p.) 4 h after the end of WBH treatment and then perfused and prefixed with PBS and 10% formaldehyde. The brain, lungs and kidneys were excised and post fixed in a solution containing 30% sucrose and 10% formaldehyde for at least 24 h. Paraffin samples were cryostat-sectioned (4 μm thick) and placed on slides coated with poly-L-lysine for TUNEL assay. TUNEL staining was done using a kit (Apo Alert DNA Fragmentation Assay kit; Clontech, BD Biosciences, Palo Alto, CA, USA) using the manufacturer's instructions. In brief, tissue slides were pretreated with 20 μg/mL of proteinase K solution for 5 min and then incubated with the reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein-conjugated deoxyuridine triphosphate (dUTP) for 1 h at 37 °C. After incubation, the sections were washed with PBS, and their nuclei were co-stained with 4,6-diamidino-2-phenylindole (DAPI) using DAPI-containing mounting medium (Vectashield R; Vector Laboratories, Burlingame, CA, USA), and subsequently analyzed using a fluorescent microscope (E800; Olympus, Tokyo, Japan) equipped with a digital camera (Coolpix 995; Olympus). Apoptosis induction efficacy was calculated as a percentage of fluorescein-positive to DAPI-stained nuclei.

2.7. Immunofluorescence staining of caspase-3, tissue factor and iNOS on hypothalamus, lung and kidney

Immunofluorescence stains for Caspase-3, tissue factor and iNOS expression were performed on hypothalamus, lung and kidney section after mice were sacrificed. 4 μm paraffin embedded hypothalamus, lung and kidney section were processed simultaneously. After rehydration, the sections were boiled in sodium citrate buffer (pH 6.0) for antigen retrieval, permeabilized and blocked in 5% BSA for 30 min at 25 °C. After removing the blocking solution and adding 200 μl primary antibody: rabbit polyclonal Anti-Caspase-3 antibody (dilution 1:200, ab44976; abcam), Rabbit monoclonal Anti-Tissue Factor antibody (dilution 1:200, ab151748; abcam), and Rabbit polyclonal Anti-iNOS antibody (dilution 1:50, ab3523; abcam), this mixture was incubated overnight at 4 °C and rinsed three times in PBS for 5 min each.Then the specimen was incubated in fluorochrome-conjugated secondary antibody: goat polyclonal secondary antibody to rabbit IgG-H&L (DyLight® 594) (dilution 1:200, ab96885; abcam), and goat anti-rabbit IgG H&L (DyLight® 488) (dilution 1:200, ab96899; abcam) in antibody dilution buffer for 2 h at room temperature in dark. After rinsing three times in PBS for 5 min each, 200 μl 4, 6-Diamidino-2-phenylindole (DAPI) staining (Molecular Probes, Eugene, OR, USA) was added to visualize nucleated cells. Slides were examined under epifluorescence on an Olympus BX60 (Tokyo, Japan) microscope.

2.8. Double immunofluorescence staining of iNOS with nitrotyrosine on hypothalamus, lung and kidney

Double immunofluorescence staining for iNOS and nitrotyrosine expression was performed on hypothalamus, lung and kidney section after mice were sacrificed. The hypothalamus, lung and kidney sections were embedded in 4-μm paraffin and processed simultaneously. After rehydration, the sections were boiled in sodium citrate buffer (pH 6.0) for antigen retrieval, permeabilized and blocked in 5% BSA for 30 min at 25 °C. The tissue slices were then incubated overnight with rabbit polyclonal Anti-iNOS (1:50, ab3523; abcam) and mouse monoclonal Anti-nitrotyrosine (1:150, ab7048, abcam). On the following day, the sections protected from light were washed and incubated with secondary antibodies: goat anti-rabbit IgG H&L (DyLight® 594) (1:200, ab96885; abcam) and goat anti-mouse IgG H&L (DyLight® 488) (1:200, ab96871; abcam) for 2 h at room temperature in dark.

2.9. Double immunofluorescence staining of TUNEL with EpCAM, caspase-3, or NeuN on hypothalamus, lung and kidney

TUNEL staining was conducted using a commercial kit (Apo Alert DNA Fragmentation Assay kit; Clontech, BD Biosciences, Palo Alto, CA, USA) by following the manufacturer's instructions. In brief, tissue slides were pretreated with 20 μg/mL of proteinase K solution for 5 min and then incubated with the reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein-conjugated deoxyuridine triphosphate (dUTP) for 1 h at 37 °C. The sections were washed with PBS, following which serum was added and incubation at 37 °C lasted for 30 min. Subsequently, these sections were incubated at 4 °C overnight after the addition of primary antibody (rabbit monoclonal Anti-NeuN, dilution 1:500, ab17748, abcam; rabbit polyclonal Anti-Caspase-3, dilution 1:200, ab44976, abcam;rabbit polyclonal Anti-EpCAM, dilution 1:100, ab71916, abcam) and then incubated at room temperature for 2 h after the addition of secondary antibody (goat anti-rabbit IgG H&L (DyLight® 594), dilution 1:200, ab96885; abcam).

2.10. Statistical analysis

Repeated-measure ANOVA was conducted to test the effect of treatment over time on each score followed by a multiple-comparison test (Scheffe's test). The Kaplan Meier analysis was used for determining the significant differences in the survival rate between control and treated groups. The Wilcoxon tests were used for evaluation of neuronal damage scores. Levels of cytokines and values of immunoassayed sections were analyzed using the Kruskal–Wallis H test, followed by several post hoc comparisons with Dunn's test. A P value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Ascorbate attenuates lethality and thermoregulatory dysfunction induced by WBH

The mice exposed to whole body heating (WBH; 41.2 °C for 1 h) may develop HS with accompanied thermoregulatory dysfunction (animals become hypothermic when exposing them to room temperature, 24 °C) and lethality. As depicted in Fig. 1a, HS-induced lethality at 24 h, 96 h, and 7 days post-WBH was attenuated by ascorbate administration (i.p.) in a dose-dependent manner ranging from 100 mg to 500 mg/kg. No mice ($n=12$) died at the dose of 500 mg/kg at 7 d post WBH. In contrast, no mice survived when saline rather than ascorbate was used. Hence, we used this dose (500 mg/kg) for ascorbate therapy in the following experiments. Meanwhile, ascorbate administration in HS mice prevented the development of thermoregulatory dysfunction which would otherwise occur, when only saline was given (Fig. 1b).

3.2. Ascorbate attenuates serum levels of pro-inflammatory cytokine TNF-α but enhances anti-inflammatory cytokine IL-10 in heat stroke mice

Fig. 2 depicts the values of serum pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) and anti-inflammatory cytokine interleukin-10 (IL-10) in NC mice, saline-treated HS mice, and ascorbate-treated HS mice. The values of serum TNF-α of saline-treated HS mice obtained at 4 h post-WBH were markedly higher than those of NC mice. Whereas, in ascorbate-treated HS mice, ascorbate administration strongly suppressed the increase in TNF-α level obtained at 4 h post-WBH. The serum levels of IL-10 in both NC and saline-treated HS mice were maintained at a negligible level during the whole course of experiment. However, ascorbate administration greatly elevated the serum levels of IL-10 in HS mice, as compared with those of NC and saline-treated HS mice.

3.3. Ascorbate attenuates heat stroke induced expressions of iNOS and nitrotyrosine in the hypothalamus, kidney and lung

As shown in Fig. 3, the numbers of iNOS-positive cells (red in DAPI blue background) in the hypothalamus (a), kidney (c) and lung (e) tissues were increased significantly at 4 h post-WBH. The heat stroke-induced increases in iNOS positive cells were reduced significantly by ascorbate administration. Furthermore, co-localization of iNOS and nitrotyrosine (a marker of nitrosative damage) in double immunofluorescence staining confirmed the occurrences of nitrosative damage in the hypothalamus (b), kidney (d) and lung (f) tissues. These data demonstrate that ascorbate attenuates the heat stroke-induced expression of iNOS and nitrosative damage in the hypothalamus, kidney and lung tissues.

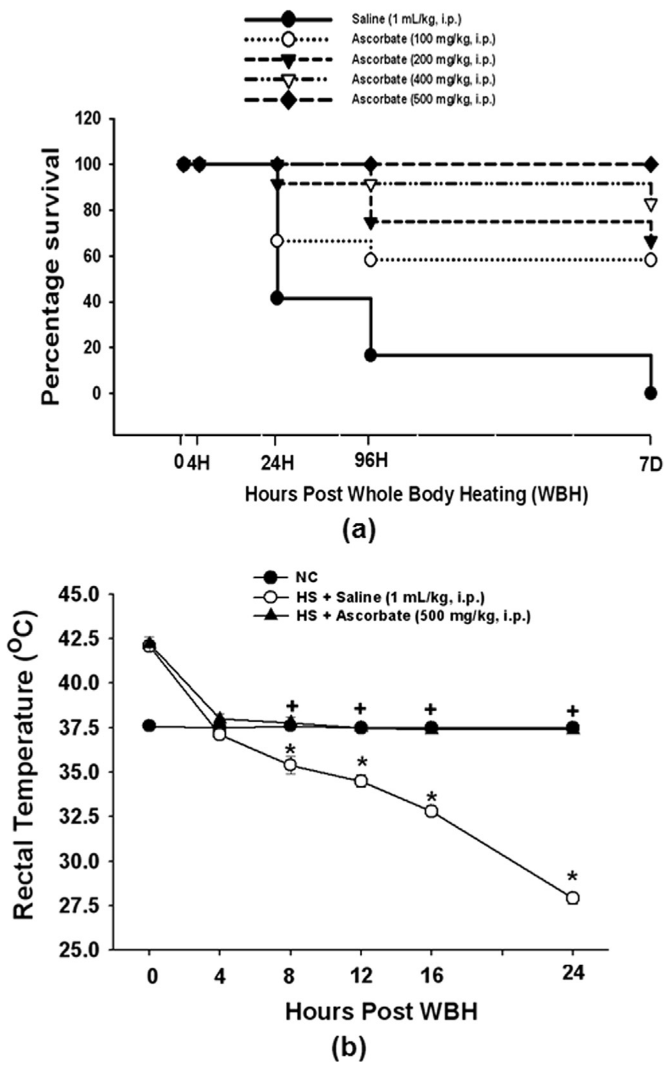

Fig. 1. Lethality (a) and thermoregulatory dysfunction (b) induced by whole-body heating (WBH, 41.2 °C for 1 h) in heat stroke (HS)-treated mice injected with saline or ascorbate (500 mg/kg, i.p.), as compared with normothermic control (NC). $^{*}P<0.05$ compared with the corresponding values in saline-treated HS mice. $^{+}P<0.05$ compared with the values in NC mice.

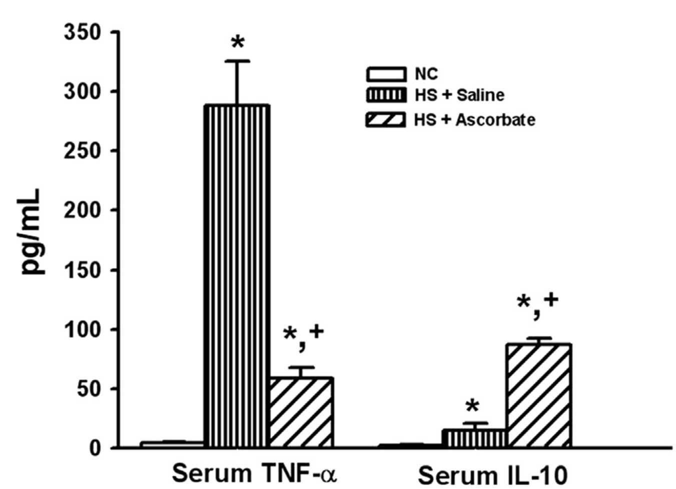

Fig. 2. Effects of whole-body heating (WBH, 41.2 °C for 1 h) on serum levels of TNF-α and IL-10 in heat stroke (HS)-treated mice injected with saline or ascorbate (500 mg/kg, i.p.), as compared with normothermic control (NC).$^{*}P<0.05$ compared with the corresponding values in NCs.$^{+}P<0.05$ compared with values in saline-treated HS mice.

3.4. *Ascorbate attenuates heat stroke-induced expressions of tissue factor in the hypothalamus and kidney*

Tissue factor is the primary initiator of the coagulation cascade. The numbers of tissue factor-positive cells in the hypothalamus (Fig. 4a) and kidney (Fig. 4b) were increased significantly at 4 h post-WBH. The heat-induced increases in tissue factor-positive cells were reduced significantly by ascorbate administration.

3.5. *Ascorbate attenuates heat stroke induced cell apoptosis in the lung and kidney*

Fig. 5 shows an absence of or only a few scattered TUNEL-positive cells in normothermic control (NC) mice. TUNEL-positive cells, indicative of apoptotic cell death, were markedly increased in lung (Fig. 5a) and kidney (Fig. 5c) tissues at 4 h post-WBH. The heat-induced increases in TUNEL-positive cells in the lung and kidney tissues were all reduced significantly by ascorbate administration. In addition, extensive co-localization of TUNEL positive with EpCAM (epithelial cell marker) positive cells in lung (Fig. 5b) and kidney (Fig. 5d) tissues in double immunofluorescence staining suggests that heat stroke induced apoptosis mostly occurs in the parenchymal cells of lung and kidney. In brief summary, we have demonstrated that ascorbate administration in HS mice significantly attenuates heat stroke-induced apoptosis and parenchymal cell damage in the lung and kidney.

3.6. *Ascorbate attenuates heat stroke induced neuronal cell apoptosis and damage in the hypothalamus*

As shown in Fig. 6, saline-treated HS mice displayed cell body shrinkage, pyknosis of the nucleus, loss of Nissl substance, and disappearance of the nucleolus (row a).The extent of neuronal damage was scored on a scale of 1–3, in which 0 is normal, 1 indicates that approximately 30% of the neurons are damaged, 2 indicates that approximately 60% of the neurons are damaged, and 3 indicates that 100% of the neurons are damaged. The neuronal damage scores for saline-treated HS mice were 2, as compared with 1 for ascorbate-treated HS mice and 0 for NC mice. Similarly, as compared to NC, saline-treated HS mice had severer apoptosis as indicated by significantly increased number of TUNEL positive (Fig. 6b) and caspase-3 positive cells per hypothalamic section (Fig. 6c). In addition, co-localization of TUNEL positive and caspase-3 positive cells (Fig. 6d) along with TUNEL positive and NeuN (a marker of neuronal cells) positive cells (Fig. 6e) in double immunofluorescence staining strongly suggests that the hypothalamic neuronal cell damage may be attributed to cell apoptosis. In brief summary, we have demonstrated that ascorbate administration in HS mice significantly attenuates heat stroke-induced neuronal cell apoptosis and damage in the hypothalamus.

4. Discussion

In this study, we demonstrated that parenteral administration of ascorbate strongly abrogated the lethality and thermoregulatory dysfunction induced by WBH in a murine model of heat stroke. In parallel with this findings, the heat stroke-induced accelerated systemic inflammatory, coagulation responses and the resultant multiple organ injury, especially in hypothalamus, were all markedly attenuated by ascorbate administration. The mice exposed to WBH developed HS with accompanied thermoregulatory dysfunction and lethality (Fig. 1), which were attenuated by ascorbate administration in dose-dependent manner (100 mg to 500 mg/kg). No mice died at the dose of 500 mg/kg at 7 days post-WBH. Hence, we used this therapeutic dose of 500 mg/kg in the following

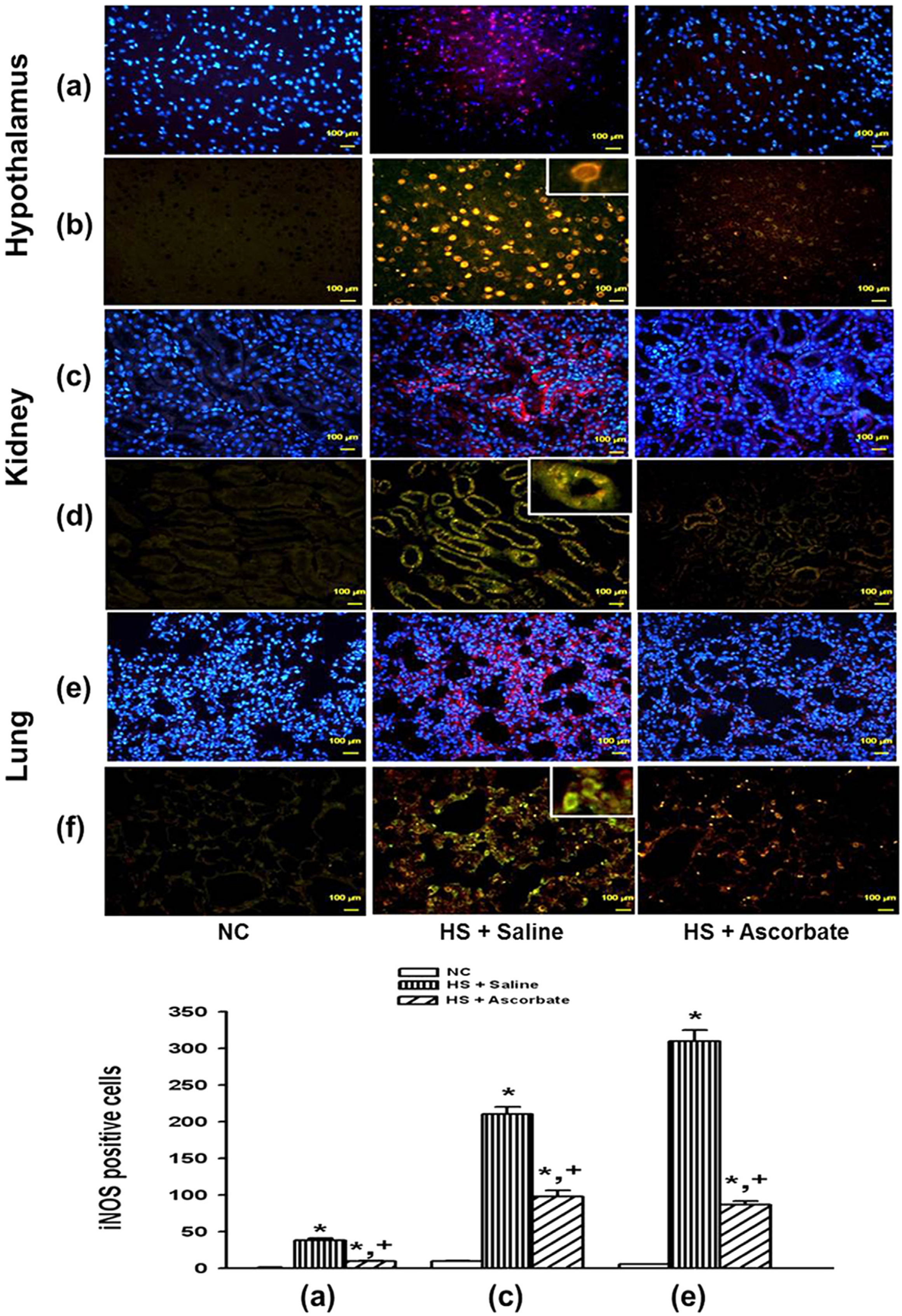

Fig. 3. Effects of WBH on the expression of iNOS in the hypothalamus (a), kidney (c), and lung (e) of saline-treated or ascorbate-treated heat stroke (HS) mice as compared with NC mice. Representative photomicrographs of double immunofluorescence showed extensive co-localization (yellow) of iNOS (red) and nitrotyrosine (green) in the hypothalamus (b), kidney (d) and lung (f) tissues. Tissue sections were obtained for iNOS and nitrotyrosine staining 4 h after whole-body heating. Data are means $\pm$ SE of six mice per group $^{*}P<0.05$ compared with the corresponding values in NCs. $^{+}P<0.05$ compared with the values in saline-treated HS mice. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

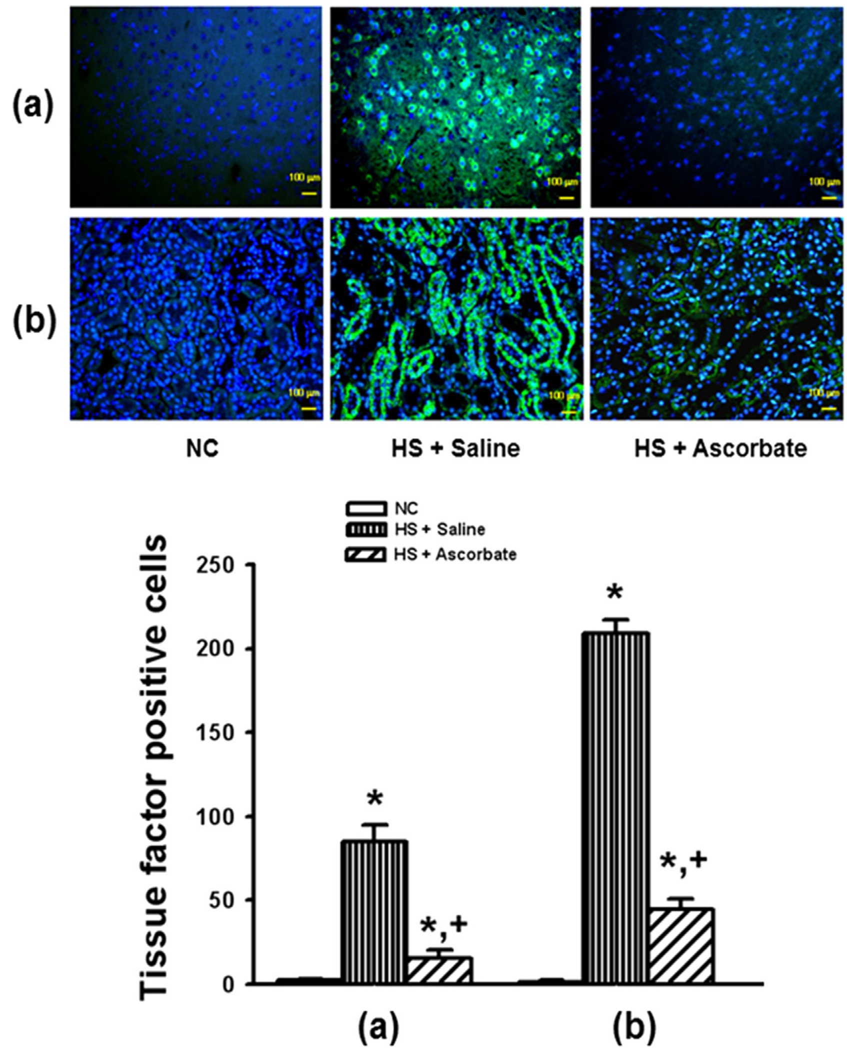

Fig. 4. Effects of whole-body heating on the expression of tissue factor per tissue section in the hypothalamus (a) and kidney (b) tissues of saline-treated or ascorbate-treated heat stroke (HS) mice as compared with NC mice. $^{*}P<0.05$ compared with the corresponding values in NCs. $^{+}P<0.05$ compared with the values in saline-treated HS mice.

experiments. Its human equivalent dose is 42 mg/kg (~2.5 g for a 60 kg adult), translated from mice by using the body surface area (BSA) normalization method [14]. A human vitamin C pharmacokinetic study showed that oral vitamin C produces plasma concentrations that are tightly controlled and that only intravenous administration of vitamin C produces high plasma concentrations that might have therapeutic activity [15]. According to the study, vitamin C at a dose of 1.25 g administered orally produced a mean peak plasma concentration of 134.8 μmol/L compared with 885 μmol/L by intravenous administration. In addition, for the maximum tolerated oral dose of 3 g every 4 h for a period of 24 h, only approximately 220 μmol/L in blood can be achieved with 90% of the dose remaining in the intestine and consequent discomfort. For the physiological dose of 200 mg, an amount that can be obtained from vitamin C-rich foods, a peak-predicted concentration is approximately 90 μmol/L. Hence, supra-physiological plasma concentrations of vitamin C can only be reached through the parenteral administration. In critical conditions such as sepsis, severe trauma or surgical patients, a drastic reduction in circulating ascorbate levels and a general depletion of antioxidant capacity are reported [16,17] and supplementation with high doses of ascorbate is required in order to restore its blood levels to normal [18].

Recent evidence has associated the host inflammatory and hemostatic responses to heat stress with multiple organ injury and death [7]. A study in baboon suggested that inflammation, microvascular injury, thrombosis and apoptosis may play an important role in the pathogenesis of HS injury [9]. In this study, we found that HS-induced accelerated systemic inflammatory responses, as assessed by increased serum levels of pro-inflammatory cytokine TNF-α, were attenuated by ascorbate administration; whereas, anti-inflammatory cytokine IL-10 was enhanced (Fig. 2). Experiments by Hartel et al. indicated that ascorbate dose-dependently inhibited production of IL-6 and TNF-α in monocytes induced by the bacterial endotoxin lipopolysaccharide (LPS) without affecting IL-1 or IL-8 levels [19]. In addition, ascorbate, at millimolar concentrations, may inhibit NF-κB activation in endothelial cells [20] and in LPS induced neuro-inflammation in primary neuron/glia cocultures [21]. NF-κB is an important transcription factor that mediates changes in gene expression during inflammation. Therefore, ascorbate may protect against inflammation not only by virtue of their ability to scavenge free radicals but also by their ability to inhibit the activation of NF-kB and possibly other oxidant-sensitive factors such as hemeoxygenase (HO)-1, a heat shock protein [22].

It has previously been demonstrated that TNF-α and IL-1β play an important role in the induction of NO in the endotoxin-induced shock in mice, although NO synthase activity is differentially

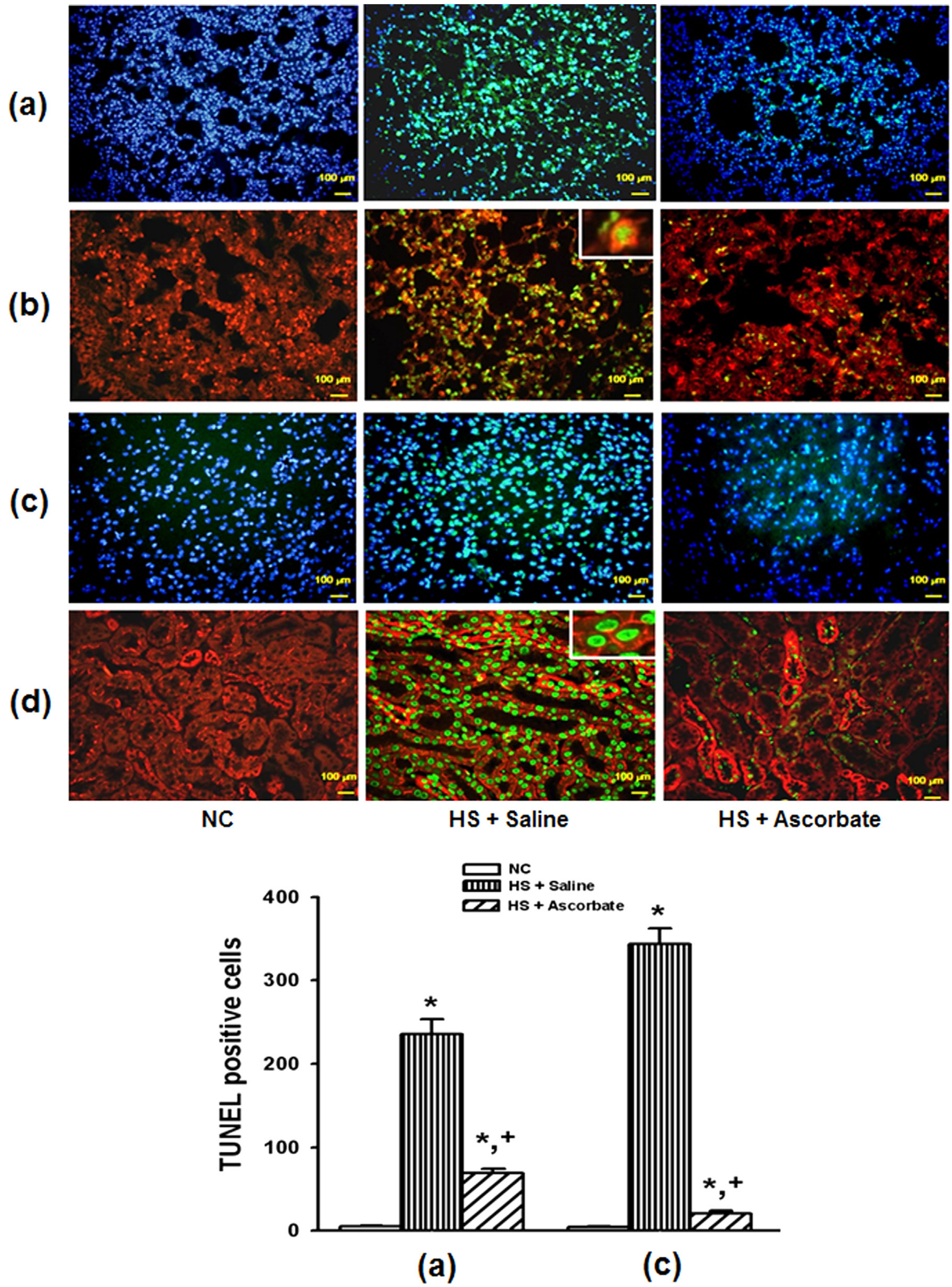

Fig. 5. Effects of whole-body heating on the expressions of TUNEL per tissue section in lungs (a) and kidneys (c) of saline-treated or ascorbate-treated heat stroke (HS) mice as compared with NC mice. Representative photomicrographs of double immunofluorescence showed extensive co-localization (yellow) of TUNEL (green) and epithelial cell marker EpCAM (red) in the tissues of lung (b) and kidney (d). Tissue sections were obtained for TUNEL and EpCAM staining 4 h after whole-body heating.$^{*}P<0.05$ compared with the corresponding values in NCs. $^{+}P<0.05$ compared with the values in saline-treated HS mice. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

expressed in various organs [23]. It is suggested that microcirculatory dysfunction due to excessive nitric oxide production by the inducible nitric oxide synthase (iNOS) is often seen in systemic inflammatory response syndromes (SIRS) [24]. In this study we found that up-regulation of iNOS and its resultant nitrosative damage are induced by heat stroke in hypothalamus, kidney and lung tissues (Fig. 3), and this detrimental effect was attenuated by ascorbate administration. In accordance with our findings, a report

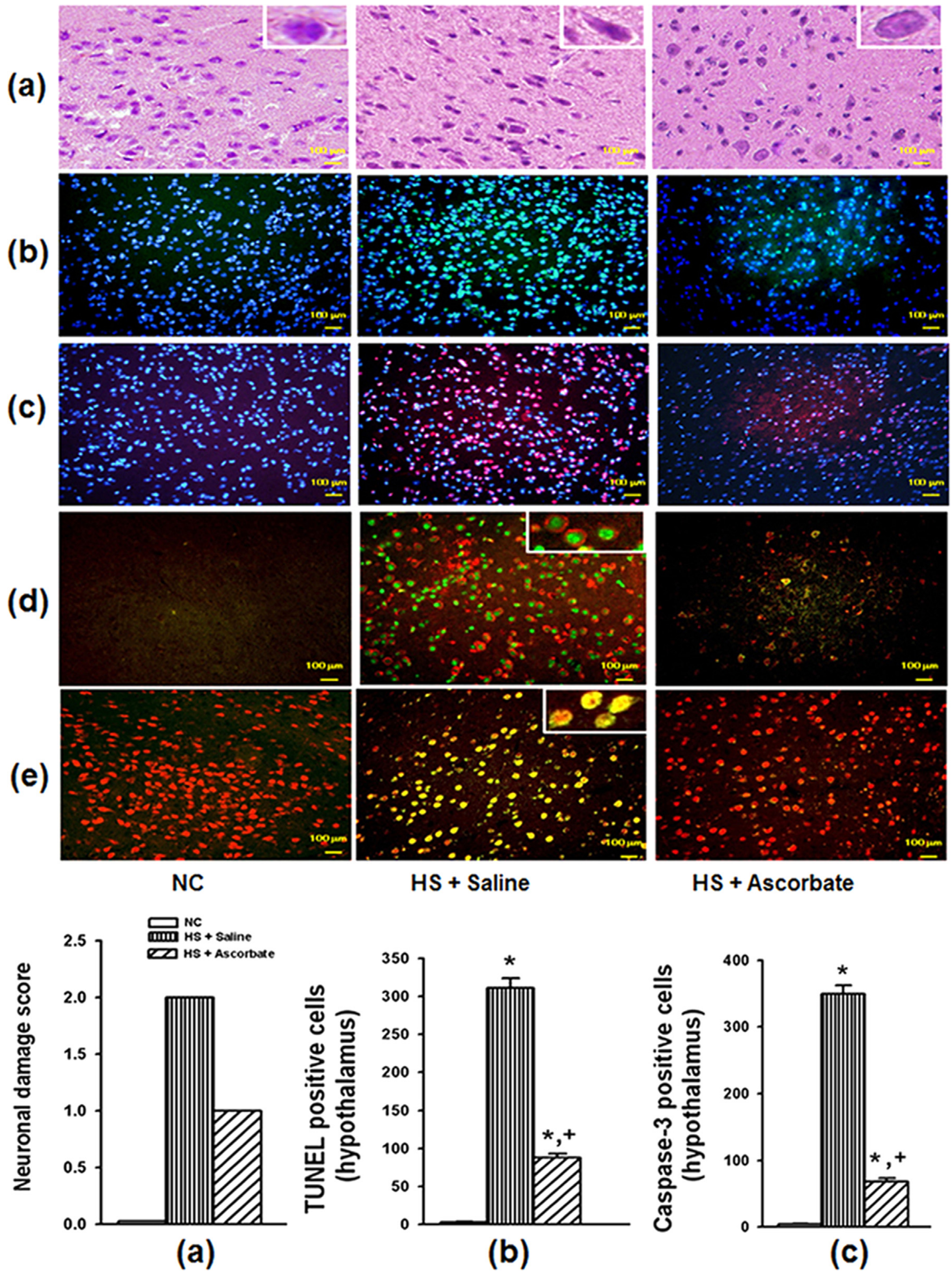

Fig. 6. Effects of whole-body heating (WBH) on neuron cell damage score (a), expression of TUNEL (b) and caspase-3 (c)per hypothalamic section of saline-treated or ascorbate-treated heat stroke (HS) mice as compared with NC mice. (a) Saline-treated HS mice displayed cell body shrinkage, pyknosis of the nucleus, loss of Nissl substance, and disappearance of the nucleolus. The neuronal damage score for saline-treated HS mice was 2, which was attenuated by ascorbate administration; (b) saline-treated HS mice showed increased TUNEL-positive cells, which were significantly reduced by ascorbate administration; (c) saline-treated HS mice showed increased caspase-3-positive cells, which were significantly reduced by ascorbate administration. Representative photomicrographs of double immunofluorescence staining of TUNEL with caspase-3 (d) or neuronal cell marker NeuN (e) showed extensive co-localization (yellow) of TUNEL (green) and caspase-3 (red) along with TUNEL and NeuN (red). * $P<0.05$ compared with the corresponding values in NCs. $^{+}P<0.05$ compared with values in HS mice treated with saline. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

has demonstrated that ascorbate protects against impaired arteriolar constriction in septic mice by inhibiting inducible nitric oxide synthase expression, and that iNOS gene knockout protects against arteriolar constriction and pressor responses identical to that provided by ascorbate [5]. Furthermore, in rats with heatstroke, pretreatment with an iNOS inhibitor aminoguanidine significantly attenuated the heatstroke-induced hyperthermia, arterial hypotension, cerebral ischemia and neuronal damage, and increased iNOS-dependent NO formation in the brain [25]. All these results suggest that, by attenuating the expressions of pro-inflammatory cytokine TNF-α and iNOS induced by heat stroke, ascorbate can ameliorate the accelerated systemic inflammatory response and prevent the subsequent endothelial dysfunction and multiple organ failure.

The mortality and neurological morbidity in heatstroke have been attributed to the host's inflammatory and hemostatic responses to heat stress [26], and it is suggested that there is an active cross-talk between vascular endothelium, inflammation and coagulation pathways in heatstroke [9]. In normal situations, the endothelium functions as an antithrombotic surface, preventing inappropriate activation of coagulation on the cell membrane. However, in systemic inflammation the endothelium becomes activated and transforms into a prothrombotic interface, which is critically involved in the detrimental cascade leading to severe coagulopathy and multiple organ failure [27]. In this study we found that ascorbate attenuates heat stroke-induced over-expression of tissue factor (TF),the pivotal initiator of inflammation-induced activation of coagulation, in the hypothalamus and kidney (Fig. 4). TF initiates coagulation by catalyzing, in a newly formed complex with FVII(a), the conversion of the zymogens FIX and X into active proteases, which in turn, enhance the activation of FX and prothrombin. Prothrombin is then converted to thrombin, which then converts fibrinogen into fibrin [28]. Histologically, TF appears to be present in all blood tissue barriers, so that coagulation can be initiated quickly once the endothelial barrier has been disrupted. Because the coagulation and inflammatory cascades function in a positive feedback loop [29] they have the potential to lead to progressive organ dysfunction and death through this vicious cycle. Therefore, it is plausible to suggest that ascorbate administration can attenuate heat stroke-induced coagulopathy by its anti-oxidant and anti-inflammatory effects. In consistent with our findings, it was reported that antioxidants inhibit LPS induction of TF expression in monocytes, macrophages, and endothelial cells [30]. Taken together, all these data support the notation that inhibition of ROS-dependent intracellular signaling may be an effective strategy for reducing TF expression and thrombotic complications associated with inflammatory diseases [31].

Multiple organ dysfunctions are believed to be the final common pathway to cause lethality in critical conditions such as heat stroke or sepsis. In the present study, we found that heat stroke causes multiple organ injury and apoptotic cell death, as indicated by significantly increased TUNEL-positive cells, and which were attenuated by ascorbate administration in lung, kidney (Fig. 5) and hypothalamus (Fig. 6b).In addition, co-localization of TUNEL-positive and caspase-3 positive cells (Fig. 6d) was found in hypothalamus, confirming the mechanism of apoptosis. Caspase-3 is noted to be a crucial apoptotic protease in the final common pathway of a death receptor–initiated caspase-8 or intrinsic pathway and mitochondria initiated caspase-9 or extrinsic pathway [32]. Our findings are compatible with those of in-vitro studies, which suggested a pivotal role of ROS as intracellular mediators of hyperthermia-induced apoptosis [33]. Our results are also in accordance with those showing that vitamin C significantly decreases heat shock-mediated apoptosis in corneal cells that involves mitochondrial and death receptor pathways [34].

In this study, we focused on measuring heat stroke-induced neuronal cell damage in the hypothalamus other than lung and kidney tissues for its pivotal role in thermoregulation and control of autonomic and endocrine activity [11]. We demonstrated that ascorbate administration in heat stroke mice could significantly improve hypothalamic neuronal cell damage and apoptosis (Fig. 6). The result is in accordance with that of a recent review [10] which suggests that ischemic and oxidative damage to the hypothalamus may be responsible for heat stroke and may cause multiple organ dysfunction or failure through hypothalamic–pituitary–adrenal axis mechanisms and that the hypothermia found in heat stroke can be explained by heat-induced hypothalamic neuronal apoptosis.

Recent evidence of the clinical benefit of parenteral vitamin C administration has emerged, especially in critical care [2]. This may partly be attributed to the effect of replenishing ascorbate levels after their drastic depletion in response to oxidative stress. Additional evidence indicates that the parenteral administration of supra-physiological doses of ascorbate restores vascular function in the systemic inflammation through modulating redox signaling in endothelium [6]. In accordance with these findings, we have herein demonstrated that parenteral administration of ascorbate effectively abrogated the lethality and thermoregulatory dysfunction in murine model of heat stroke by attenuating heat stroke-induced accelerated systemic inflammatory, coagulation responses and the resultant multiple organ injury, especially in hypothalamus. Overall, the clinical benefit of the rapid replenishment of ascorbate levels through the parenteral administration of vitamin C is anticipated particularly in critical care, in which systemic inflammatory responses dominate and death are often inevitable if overwhelming oxidative stresses and inflammatory responses are not controlled. A recent phase I safety trial of intravenous ascorbic acid in patients with severe sepsis has proved its safety, good tolerance and positive impacts in the extent of multiple organ failure and biomarkers of inflammation and endothelial injury [35].

5. Conclusion

In this study, we have demonstrated that parenteral administration of supra-physiological doses of ascorbate can abrogate the lethality and thermoregulatory dysfunction in a murine model of heat stroke by attenuating HS-induced accelerated systemic inflammatory, coagulation responses and the resultant multiple organ injury, especially in hypothalamus. In conclusion, our findings support the hypothesis and notion that supra-physiological doses of ascorbate may have therapeutic use in critical care. Future studies are warranted to determine the exact clinical benefit.

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