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Experimental Neurology 199 (2006) 67 – 76
Experimental Neurology
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Infusion of human umbilical cord blood cells protect against cerebral ischemia and damage during heatstroke in the rat

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Received 12 July 2005; revised 21 October 2005; accepted 18 November 2005
Available online 6 January 2006

Abstract

Intravenously delivered human umbilical cord blood cells (HUCBC) have been previously shown to improve both morphologic and functional recovery of heat-stroked rats. To extend these findings, we examined both the morphologic and functional alterations in the presence of HUCBC or human peripheral mononuclear cells (PBMC) 24 h before initiation of heatstroke. Anesthetized rats, 1 day before the initiation of heatstroke, were divided into three major groups and given the following: (a) serum-free lymphocyte medium (0.3 ml) intravenously; (b) PBMC (5×10^6 in 0.3 ml serum-free lymphocyte medium); or (c) HUCBC (5×10^6 in 0.3 ml serum-free lymphocyte medium). Another group of rats were exposed to room temperature (26°C) and used as normothermic controls. In vehicle-treated heatstroke rats, their mean arterial pressure, cerebral blood flow, and brain PO_2 were all lower than in normothermic controls after the onset of heatstroke. However, their body temperatures and striatal levels of inducible nitric oxide synthase (iNOS)-dependent NO, ischemia and damage markers (e.g., glycerol, glutamate, and lactate/pyruvate ratio), and neuronal damage in the striatum were all greater. The heatstroke-induced arterial hypotension, cerebral ischemia and hypoxia, and increased levels of iNOS-dependent NO in the striatum were all significantly reduced by pretreatment with HUCBC, but not with PBMC. Moreover, HUCBC were localized by immunohistochemistry and PCR analysis in the injured brain structures and spleen. These findings indicate that HUCBC transplantation, in addition to having therapeutic values, can be a good choice for preventing heatstroke occurrence.
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Keywords: Human umbilical cord blood cells; Heatstroke; Nitric oxide; Glycerol; Glutamate; Brain

Introduction

Heatstroke afflicts many humans engaged in severe exercising, firefight, or some military or mining activities (Halle and Repasy, 1987; Knochel, 1989; Simon, 1993). Many predisposing diseases (such as cardiovascular disease, diabetes mellitus, alcoholism, and impaired sweat production) or factors (such as salt and water depletion and fever after immunization) may increase susceptibility to heatstroke (Dahmash et al., 1993; Knochel, 1989) Unless promptly recognized and treated, hyperthermia, central nervous system dysfunctions (such as restlessness, delirium and coma), and multiple organ failure may occur and result in high rate of mortality (Bouchama and Knochel, 2002).

It is well known that the lactate/pyruvate ratio is a marker of cell ischemia, whereas glycerol is a marker of how severely cells are affected by ongoing pathology (Hillered and Persson, 1999; Hillered et al., 1990, 1998). Excessive accumulation of glutamate has been shown in ischemic brain tissue (Nilsson et al., 1996; Persson and Hillered, 1992; Ungerstedt, 1997). Indeed, when the animals are exposed to hot environment, cerebral ischemia and hypoxia which occurred during heatstroke were associated with an increased production of glycerol, lactate/pyruvate ratio, glutamate, and inducible nitric oxide synthase (iNOS)-dependent NO in the brain (Chang et al., 2004; Chen et al., 2005; Kuo et al., 2003). In addition, the PO_2 in rat brain was decreased after the onset of heatstroke onset. Thus, it appears that excessive accumulation of glycerol, glutamate, lactate/pyruvate ratio, and iNOS-dependent NO in the brain may be secondary to cerebral ischemia, hypoxia, and/or injury in the rat.

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0014-4886/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2005.11.015

It is estimated that approximately 2% of the human umbilical blood cells (HUCBC), similar to the percentage observed in bone marrow, are stem cells capable of reconstituting blood lineages (Bender et al., 1991; Ho et al., 1996; Nieda et al., 1997; Wu et al., 1999). Intravenously delivered HUCBC found in the brain 4 weeks after middle cerebral artery occlusion in the rat were excessively localized to the ischemic hemispheres (Wang et al., 2004). HUCBC have emerged as an alternative to bone marrow since they possess greater availability, lower risk of mediating viral transmission, and weaker immunogenicity (Lewis, 2002). Accumulating evidence has demonstrated that HUCBC transplantation is a promising new therapeutic method against neurodegenerative diseases, such as stroke, traumatic brain injury, and spinal cord injury as well as blood diseases (Chen et al., 2001b; Lu et al., 2002; Saporta et al., 2003; Wang et al., 2004; Willing et al., 2003). More recently, we have also demonstrated that HUCBC therapy may resuscitate rats with heatstroke by reducing circulatory shock, and cerebral NO overload and ischemic injury; central delivery of HUCBC seems superior to systemic delivery of HUCBC in resuscitating rats with heatstroke (Chen et al., 2005). However, to our knowledge, evidence is not available about the preventive effects of HUCBC administered before the initiation of cerebral ischemia and injury.

To deal with the question, the present study was designed to investigate whether an early in exposure to HUCBC treatment could still promote attenuation of both the morphologic and functional alterations which occurred during heatstroke in the rat. We attempted to elucidate whether HUCBC pretreatment could be a good choice for preventing heatstroke occurrence.

Methods

Animals

Adult Sprague–Dawley rats (weight, 287 ± 16 g) were obtained from the Animal Resource Center of the National Science Council of the Republic of China (Taipei, Taiwan). The animals were housed 4 in a group at an ambient temperature (Ta) of 22 ± 1°C, with a 12-h light/dark cycle. Pellet rat chow and tap water were available ad libitum. All protocols were approved by the Animal Ethics Committee of the Chi-Mei Medical Center (Tainan, Taiwan) in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act. Adequate anesthesia was maintained to abolish the corneal reflex and pain reflexes induced by tail pinching throughout all experiments (approximately 8 h) by a single intraperitoneal dose of urethane (1.4 g/kg body weight). At the end of the experiments, control rats and any rats that had survived heatstroke were killed with an overdose of urethane.

Surgery and physiological parameter monitoring

The right femoral artery and vein of rats were cannulated with polyethylene tubing (PE 50), under urethane anesthesia, for blood pressure monitoring and drug administration. Core temperature (Tco) was monitored continuously by a thermocouple, while both mean arterial pressure (MAP) and heart rate (HR) were continuously monitored with a pressure transducer.

Induction of heatstroke

The Tco of the anesthetized animals were maintained at about 36°C with an infrared light lamp except the heat stress experiments. Heatstroke was induced by putting the animals in a folded heating pad of 43°C controlled by circulating hot water. The instant in which the MAP dropped irreversibly from the peak was taken as the onset of heatstroke (Kao et al., 1994; Yang and Lin, 1999). After the onset of heatstroke, the heating pad was removed, and the animals were allowed to recover at room temperature (26°C). Our pilot results showed that the latency for onset of heatstroke in vehicle-treated rats was found to be 68 ± 2 min (n = 8). Therefore, in the following heatstroke groups of rats, all animals were exposed to 43°C for exactly 68 min and then allowed to recover at room temperature (26°C).

Experimental groups

Animals were assigned randomly to one of following 4 groups. One group of rats (n = 24), treated with an i.v. dose of vehicle solution (0.3 ml serum-free lymphocyte medium) per rat 24 h before the initiation of heat exposure, were exposed to an ambient temperature (Ta) of 26°C, and their physiological parameters were continuously recorded for up to 480 min (or at the end of the experiments). This group of animals was used as normothermic controls. The second group of rats (n = 24), treated with an i.v. dose of vehicle solution (0.3 ml serum-free lymphocyte medium) per rat 24 h before the initiation of heat exposure (Ta 43°C for 68 min), were used as vehicle-treated heatstroke controls. The third group of rats (n = 24) were treated with an i.v. dose of PBMC (5×10^6 in 0.3 ml serum-free lymphocyte medium) 24 h before the initiation of heat exposure (Ta 43°C). The fourth group of rats were (n = 24) treated with an i.v. dose of HUCBC (5×10^6 in 0.3 ml serum-free lymphocyte medium) 24 h before the initiation of heat exposure (Ta 43°C). The last 3 groups of rats were exposed to heat exposure (43°C) for exactly 68 min to induce heatstroke and then allowed to recover at room temperature (26°C). Both physiological parameters and survival time (interval between the initiation of heat exposure and animal death) were observed up to 480 min (or at the end of the experimentation).

Preparation of PBMC and HUCBC

Human PBMC and HUCBC were obtained from freshly collected buffy coat fraction from healthy donors at the Tainan Blood Bank Center (Tainan, Taiwan) and Chi-Mei Medical Center (Tainan, Taiwan), respectively. It was isolated by centrifugation over a Fricoll-Pague (Famacia, Uppsala, Sweden) density gradient at 400 g for 30 min at room temperature in a Sowall RT600 B (Du Pont, DE). The cells collected at the interface were washed thrice with serum-free Roswell Park

Memorial Institute (RPMI)-1640 (GIBCO, BRL, Grand Island, NY) and subsequently resuspended in serum-free lymphocyte medium (GIBCO, BRL). The PBMC at a concentration of 5×10^6 cells in 0.3 ml was prepared and stored in a 37°C incubator. For intravenous administration, a 26-gauge needle was inserted into the tail vein, and cells (0.3 ml) were delivered over a 1-min period.

Measurements of cerebral blood flow, brain O_2, and brain temperature

A 100-μm diameter thermocouple and two 230-μm fibers were attached to the oxygen probe. This combined probe measures oxygen, temperature, and microvascular blood flow. The measurement requires OxyLite™ and OxyFlo™ instruments. OxyLite 2000 (Oxford optronix Ltd., Oxford, UK) is a 2-channel device (measuring PO_2 and temperature at two sites simultaneously), whereas OxyFlo 2000 is a 2-channel Laser Doppler perfusion monitoring instrument. The OxyLite has been designed to operate in conjunction with OxyFlo. The combination of these 2 instruments provides simultaneous tissue blood flow, oxygenation, and temperature data. Under urethane anesthesia, the animal was placed in a stereotaxic apparatus, and the combined probe was implanted into the striatum using the atlas and coordinates of Paxinos and Watson (1982). The detailed procedures for measurement of brain temperature, PO_2, temperature were described previously (Chang et al., 2004; Chou et al., 2003).

Measurement of extracellular glutamate, glycerol, and lactate/pyruvate ratio in the striatum

Animals were anesthetized with urethane administered intraperitoneally. The animal's head was mounted in a stereotaxic apparatus (Davis Kopf Instruments) with the nose bar positioned 3.3 mm below the horizontal line. Following a midline incision, the skull was exposed, and a burr hole was made in the skull for the insertion of a dialysis probe (4 mm in length, CMA/12, Carnegie Medicine, Stockholm, Sweden). The microdialysis probe was stereotaxically implanted into the striatum according to the atlas and coordinates of Paxinos and Watson (1982). The detailed procedures for measurements of cellular ischemic and damage markers were described previously (Chou et al., 2003).

Extracellular NO monitoring

A microdialysis probe (CMA20; Carnegie Medicine, Stockholm, Sweden) with a 4-mm-long dialysis membrane was vertically implanted into the striatum. A Ringer's solution (0.860 g NaCl, 0.30 g KCl, 0.033 g $CaCl_2$ per 100 ml) was perfused through the microdialysis probe at a constant flow (2.0 ml/min). After 6 h of stabilization, the dialysates from the striatum were collected at 20-min intervals. The NO concentrations in the dialysates were measured with the Eicom ENO-20 NO analysis system (Eicom, Kyoto) (Togashi et al., 1998).

Neuronal damage score

At the end of the experiments, animals were killed by an overdose of urethane, and the brains were fixed in situ and left in the skull in 10% neutral-buffered formalin for at least 24 h prior to removal from the skull. The brain was removed and embedded in paraffin blocks. Serial (10 μm) sections through the striatum, hippocampus, hypothalamus, and frontal cortex were stained with hematoxylin and eosin for microscopic evaluation. The extent of cerebral neuronal damage in different brain structures was scored on a scale of 0–3, modified from the grading system of Pulsinelli et al. (1982a) in which 0 is normal, 1 indicates 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 without the examiner knowing the experimental conditions.

Immunohistochemical staining

Rats were killed with intravenous urethane (2.8 g/kg) and were transcardially perfused with heparinized 0.05 mol/l phosphate-buffered saline (PBS) followed by ice-cold 15% sucrose in PBS. The brains were rapidly removed and frozen in liquid nitrogen. Coronal brain sections (5 μm thick) were cut on a cryostat and were thaw mounted on gelatin-coated slides. The endogenous peroxidase was blocked with 10% MeOH/3% H_2O_2/sodium phosphate buffer solution mixture for 30 min at room temperature. Preincubation with 2% normal goat serum (NGS) containing 0.2% Triton X-100 (Sigma Chemical Co.) was carried out at room temperature for 1–2 h to block non-specific binding of immunoglobin G (IgG). Sections were incubated with commercially available rabbit anti-NOS antiserum (1:50) diluted in 0.2% Triton X-100, 1% azide (Sigma)/PBS at 4°C overnight, then rinsed with PBS for 30 min, and incubated in biotinylated goat anti-rabbit IgG (1:500) for 1–2 h. After several rinses with PBS, sections were incubated in AB mixture (avidin-biotin complex, 1:200; Vectastatin) for 1–5 h, incubated in 3-3′-diaminobenzidine (DAB; Daceo Co., Copenhagen, Denmark) and nickel ammonium sulfate in the presence of 0.003% H_2O_2, and then mounted on gelatinized slides. The specificity of each anti-serum was demonstrated by the absence of stain when diluted primary anti-serum was preabsorbed with the respective antigen or was replaced by normal serum. For the negative control, sections were incubated with heat-denatured primary antibody. The results of these immunocytochemistry controls were consistently negative.

Polymerase chain reaction analysis

DNA was obtained from the brain and other organs of half of the animals for each group. Polymerase chain reaction (PCR) was performed for the human glycerol-3-phosphate dehydrogenase (HG3PDH) gene using primers (sense: 5′-GGCTGGGAC-T-CATGGAGAT-3′; and antisense: 5′-CGGGTAAGTCGTTG-A-GAAAG-3′). Nested PCR was performed with primers (sense: 5′-TCTTGGAGAGCTGTGG-TGTTG-3′; antisense:

5′-GTTACCTGAAAGGACTGC-3′). Products were resolved on 3.5% polyacrylamide gels and visualized by silver staining.

Immunohistochemistry

Autofluorescence was first quenched using the method of Vendrame et al. (2004), after which the sections were incubated with mouse monoclonal antibody against human nuclei (HuNu, Chemicon, Inc.), followed by fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Alexa Molecular Probe). DAPI staining (Molecular Probes, Eugene, Ore) was performed to visualize nucleated cells. Slides were examined under epifluorescence on an Olympus BX60 microscope.

Statistical analysis

Data are presented as means ± SEM. Repeated measures ANOVA was conducted to test the treatment by time interactions and the effect of treatment over time on each score. The Duncan's multiple-range test was used for post hoc multiple comparison among means. The Wilcoxon tests were used for evaluation of neuronal damage scores. The Wilcoxon test converts the scores or values of a variable to ranks, requires calculation of a sum of the ranks, and provides critical values for the sum necessary to test the null hypothesis at a given significant level. These data were presented as "median", followed by first (Q_1) and third (Q_3) quartile. A P value less than 0.05 was calculated as statistical significance.

Results

HUCBC pretreatment extends latency and improves survival during heatstroke

Table 1 summarizes the latency and survival time for vehicle-pretreated, PBMC-pretreated, and HUCBC-pretreated rats during heatstroke. It can be seen from the table that the latency and the survival time values were found to be 68–70 min (n = 8) and 18–22 min (n = 8) respectively for vehicle-treated heatstroke rats. The values of latency and survival time for PBMC-pretreated heatstroke rats were not distinguishable from those of the vehicle-pretreated heatstroke rats. However, as compared with those of vehicle-pretreated or PBMC-pretreated heatstroke rats, the values for both the latency and survival time were significantly greater in those of HUCBC-pretreated heatstroke rats (79–81 min for latency and 118–138 min for survival time).

Table 1
Effects of heat exposure (HE; Ta = 43°C for 68 min) on survival time in rats treated with medium, in rats treated with peripheral blood mononuclear cells (PBMC), and in rats treated with human umbilical cord blood cells (HUCBC) 24 h before the initiation of HE

Treatments	Latency (min)	Survival time (min) [a]
1. Normothermic controls	>480 (8)	>480 (8)
2. Vehicle-treated heatstroke controls	68 ± 2 (8) *	20 ± 2 (8) *
3. PBMC (5×10^6, i.v.)-treated heatstroke rats	66 ± 3 (8) *	21 ± 2 (8) *
4. HUCBC (5×10^6, i.v.)-treated heatstroke rats	81 ± 2 (8) *,**	123 ± 15 (8) *,**

[a] All drug-treated or vehicle-treated heatstroke groups, exposed to 43°C, had HE withdrawn at 68 min and then allowed to recover at room temperature (26°C). Except normothermic controls, data are means ± SEM followed by number of animals used in parentheses. Group 1 was terminated about 480 min after the initiation of experiment (or at the end of experiment) by overdose of an anesthetic, so they should survive more than 480 min.

* $P < 0.05$ compared with group 1.

** $P < 0.05$ compared with group 3 (ANOVA followed by Duncan's test).

HUCBC pretreatment attenuates hypotension and cerebral ischemia and damage during heatstroke

Both Figs. 1 and 2 showed the effect of heat exposure (43°C for 68 min) on MAP, NO, CBF, Brain PO_2, Tb, Tco, HR, and striatal levels of glutamate, glycerol, lactate/pyruvate ratio in rats pretreated with PBMC (5×10^6 in 0.3 ml serum-free lymphocyte medium), and in rats pretreated with HUCBC (5×10^6 in 0.3 ml serum-free lymphocyte medium) 24 h before the initiation of heat exposure. As shown in these two figures, 12 min after the termination of heat exposure in the PBMC-treated group, all the MAP, CBF, brain PO_2, and HR values were significantly lower than those of the normothermic controls ($P < 0.05$). On the other hand, the values of Tb, Tco, and levels of glutamate, glycerol, lactate/pyruvate ratio, and NO in the extracellular fluids of the striatum in the PBMC-treated group were significantly higher 12 min after the termination of 68-min heat exposure than in those of the normothermic controls. Heatstroke-induced arterial hypotension, cerebral ischemia and hypoxia, and increased levels of glutamate, glycerol, lactate/pyruvate ratio, and NO in the extracellular levels of striatum were significantly attenuated by pretreatment with HUCBC 24 h before the initiation of heat exposure.

HUCBC pretreatment attenuates iNOS expression and neuronal damage score in striatum during heatstroke

In separate experiments, 12 min after the onset of heatstroke, animals were killed for determination of both iNOS expression and neuronal damage score in striatum. The data are summarized in Table 2. After the onset of heatstroke, animals pretreated with PBMC displayed higher values of striatal neuronal damage score and iNOS immunoreactivity compared with those of normothermic controls. In addition, histopathological verification revealed that heatstroke caused cell body shrinkage, pyknosis of the nucleus, loss of Nissl substance, disappearance of the nucleolus (Fig. 3B), and overexpression of iNOS (Fig. 4B) in the striatum of the PBMC-pretreated rats. Empty spaces around damaged neurons in Fig. 3B probably represent swollen astrocytic foot processes (Pulsinelli et al., 1982b,c). However, with the HUCBC pretreatment neuroprotection was ensured (Figs. 3C and 4C).

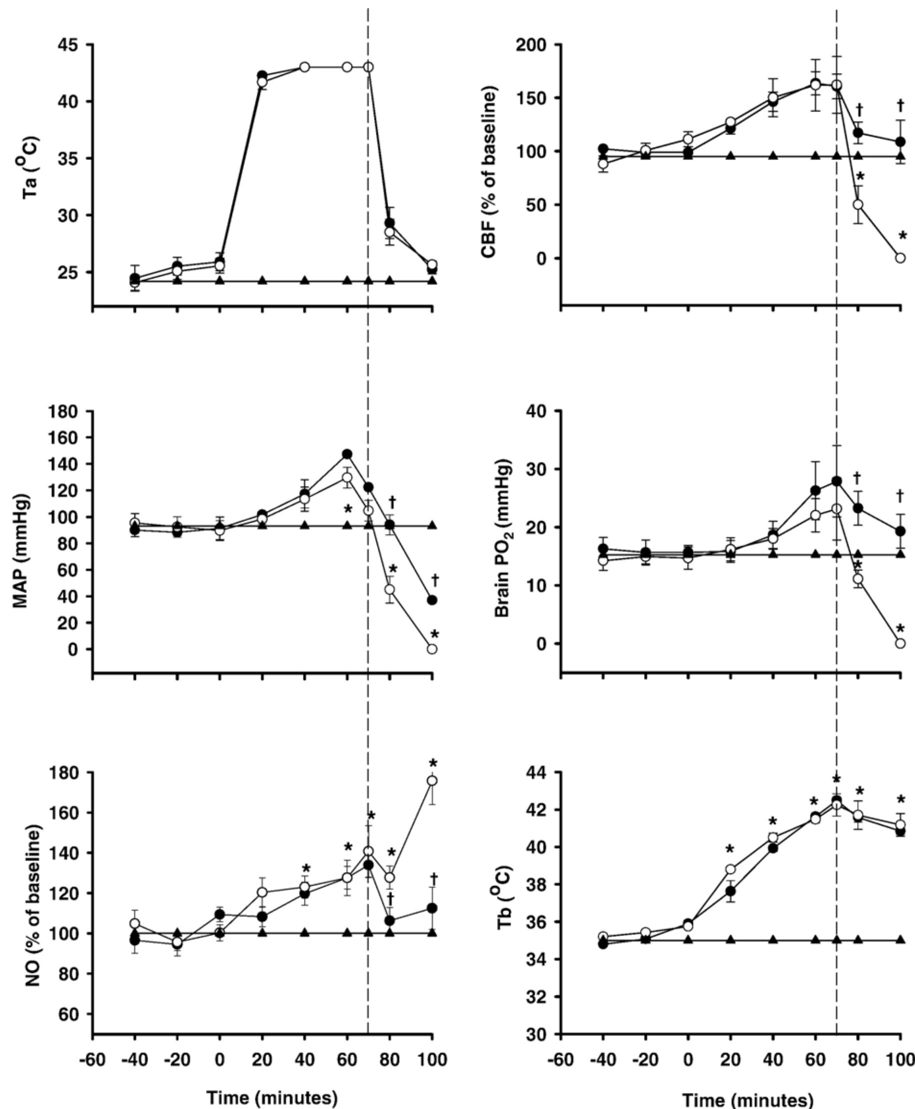

Fig. 1. Effects of heat stress (Ta 43°C for 68 min) on core temperature (Tco), mean arterial pressure (MAP), and levels of nitric oxide (NO), cerebral blood flow (CBF), PO_2, and temperature (Tb) in the striatum. Open circles, values at Ta of 43°C in 8 rats treated with PBMC (5×10^6/0.3 ml, i.v.) 24 h before the initiation of heat exposure. Solid circles, values at Ta of 43°C in 8 rats treated with HUCBC (5×10^6/0.3 ml, i.v.) 24 h before the initiation of heat exposure. Another 8 rats were used as normothermic controls (solid triangles). Points represent means ± SEM. $^*P < 0.05$ compared with those of normothermic controls; $^{\dagger}P < 0.05$ compared with PBMC-treated group (at 43°C) (ANOVA followed by Duncan's test). The dotted line denotes the onset of heatstroke.

Delivered cells were localized to brain as determined by immunohistochemistry and PCR during heatstroke

Human nuclei immunoreactive cells were detected in the striatum (Fig. 5) and other brain structures of animals injected with 5×10^6 cell dose 24 h before the initiation of heat stress. To confirm the organ distribution of HUCBC, the animals were killed after 68-min heat exposure plus 12-min room-temperature exposure after termination of heat exposure. PCR analysis revealed that HG3PDH only in spleen (data not shown) and brain (Fig. 6).

Discussion

Pretreatment (present results) or posttreatment (Chen et al., 2005) with HUCBC significantly attenuates the arterial hypotension, cerebral ischemia and hypoxia, and increased levels of ischemia and damage markers in the brain during heatstroke. These findings demonstrate that HUCBC is effective for prevention and repair of circulatory shock and ischemic damage in the brain during heatstroke by reducing iNOS-dependent NO formation in the brain. However, treatment with PBMC fails to produce any significant protection. We attribute the discrepancy between PBMC and HUCBC treatments to the lack of pleuropotential of PBMCs.

It has been shown that systemic delivery of either whole bone marrow (Chen et al., 2001a; Li et al., 2000) or umbilical cord blood cells (Chen et al., 2001b; Lu et al., 2002; Saporta et al., 2003) can produce behavioral recovery after stroke, traumatic brain injury, or spinal cord injury. The results obtained here have further shown that HUCBC have an ability to prevent circulatory shock and brain ischemic damage during the heatstroke

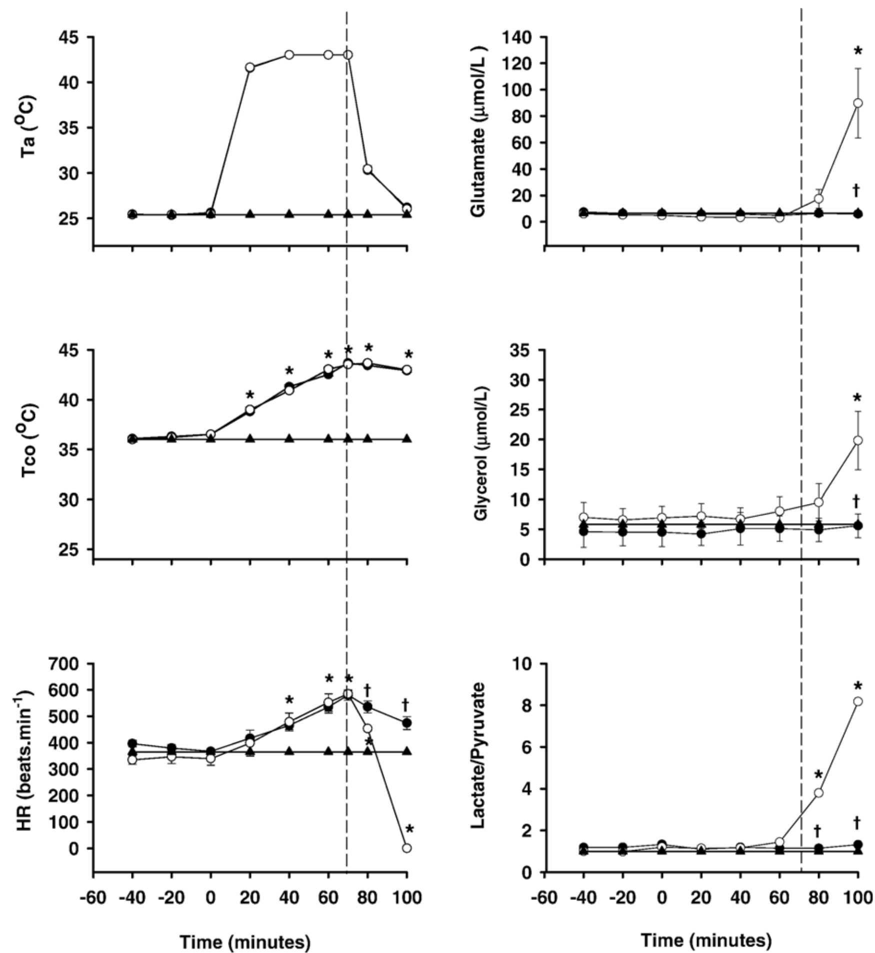

Fig. 2. Effects of heat stress (Ta 43°C for 68 min) on Tco, HR, and levels of glutamate, glycerol, and lactate/pyruvate ratio in the extracellular fluids of the striatum. Open circles, values at Ta of 43°C in 8 rats treated with PBMC (5×10^6/0.3 ml, i.v.). Solid circles, values at Ta of 43°C in 8 rats treated with HUCBC (5×10^6/0.3 ml, i.v.). PBMC or HUCBC solution was administered 24 h before the initiation of heat exposure. Another 8 rats were used as normothermic controls (solid triangles). *$P < 0.05$ compared with those of normothermic controls; †$P < 0.05$ compared with PBMC-treated heatstroke group (at 43°C) (ANOVA followed by Duncan's test). The dotted line denotes the onset of heatstroke.

and, moreover, that HUCBC administered right after the onset of heatstroke is still effective for improving circulatory shock and ischemic damage in the brain (Chen et al., 2005).

Table 2
Effects of heat exposure (43°C for 68 min) plus 12-min room-temperature (26°C) exposure on both the iNOS immunoreactivity and neuronal damage score values of the striatum from normothermic controls

Treatments	iNOS immunoreactivity (0–3)	Neuronal damage score (0–3)
1. Normothermic controls	(0, 0.75)	(0, 0.75)
2. PBMC-treated heatstroke rats	2(2, 2) *	2(2, 2) *
3. HUCBC-treated heatstroke rats	1(0, 1) **	1(0.25, 0.75) **

PBMC-treated heatstroke rats, and HUCBC-treated heatstroke rats.
Values represent the median with the first and third quartile in parentheses of eight rats per group. For determination of iNOS immunoreactivity and neuronal damage score, animals were killed after 68-min heat exposure plus 12-min room-temperature exposure after termination of heat exposure. The data were evaluated by a Wilcoxon signed rank test followed by the Duncan's test when appropriate.
* $P < 0.05$ compared with group 1.
** $P < 0.05$ compared with group 2 (ANOVA followed by Duncan's test).

Subjection of conscious rats to a 4 h heat stress (38°C) resulted in profound hyperthermia (41.86°C), salivation, and behavioral prostration (Sharma et al., 1997). Post mortem examination showed gastric hemorrhage, increased blood–brain barrier permeability to Evans blue, and brain edema. Light microscopy showed many distorted neurons and gliosis in several brain regions of heat-stressed rats, evidenced by degeneration of Nissl substance in the neuronal cytoplasm and upregulation of glial fibrillary acidic protein (GFAP) immunoreactivity, a marker of astrocytes. Electron microscopy demonstrated perivascular edema, dark and distorted neurones, swollen astrocytes, damage of postsynaptic dendrites, and degeneration of myelin in many parts of the brain. Subjection of conscious rabbits to heat stress (40°C) displayed congestion in leptomeninges and the choroid plexuses of the ventricles at the onset of heatstroke (Shih et al., 1984). In addition, degeneration of neurons, as well as pyknosis and swelling of the dendrites with replacement by proliferating microglia, was noted in different brain structures. When heatstroke was induced in the urethane-anesthetized rats, gliosis, neuronal loss and degeneration, cell body shrinkage, pyknosis of the nucleus, loss of Nissl

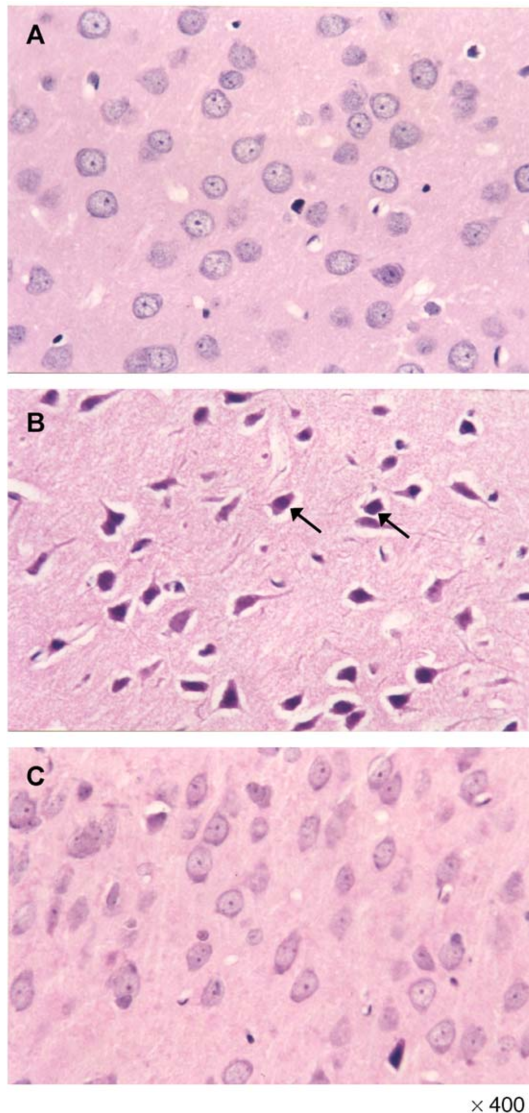

Fig. 3. Histological examination of neuronal damage. The photomicrographs of the striatum in a normothermic control rat (A), a heatstroke rat treated with PBMC (5×10^6/0.3 ml, i.v.) (B), or a heatstroke rat treated with HUCBC (5×10^6/0.3 ml, i.v.) (C) 24 h before the initiation of heat stress. Twelve minutes after 68-min heat exposure, the striatum of the rat treated with PBMC showed cell body shrinkage, pyknosis of the nucleus, loss of Nissl substance, and disappearance of nucleolus (arrows in B). Empty spaces around damaged neurons in panel B probably represent swollen astrocytic foot processes. However, treatment with HUCBC, neuronal damage was reduced, as shown in panel C. The animals were killed at 12 min after the termination of heat exposure or the equivalent time for the normothermic controls. ×400.

substance, and disappearance of the nucleolus in neuronal cells of different parts of brain were noted during heatstroke (Chou et al., 2003; Lin et al., 1997; Liu et al., 2000; Vendrame et al., 2004; Yang and Lin, 2002). The expression of proto-oncogene c-fos was also induced in different brain regions by heat stress in the rat (Tsay et al., 1999). Thus, it appears that pathological changes such as some swollen cells, disturbed tissue connection, edema, and necrotic cell death with invasion of reactive inflammatory cells (Petito et al., 1982; Pulsinelli et al., 1982b,c) occur during heatstroke. A recent MRI study further reveals a critical pathological hallmark of heatstroke involving a mixture of ischemia with edema and bleeding in the brains of patients (McLaughlin et al., 2003). Nevertheless, in the present study, we only characterize neuroprotection of HUCBC grafts against heatstroke based on the extent of cell body shrinkage and pyknosis of the nucleus according to the grading system of Pulsinelli et al. (1982a).

Intravenously delivered HUCBC found in the brain after the onset of heatstroke were localized to the ischemic hemisphere. Moreover, immunofluorescent localization of the HUCBC by human nuclei detection suggested that their numbers were large and widely distributed to different brain structures. The intravenously injected HUCBC may be following homing signals that attract them to the injured site (Chen et al., 2001a,b; Newman et al., 2003), in particular the hyperthermic brain.

Accumulating evidence has demonstrated that HUCBC transplantation is a promising new therapeutic method against neurodegenerative diseases, such as stroke, traumatic brain injury, and spinal cord injury, as well as blood diseases. In particular, intravenous administration of HUCBC can secrete therapeutic molecules, such as neurotrophic factors, which when exogenously administered by themselves are neuroprotective in stroke models (Borlongan et al., 2004; Wen et al., 2003); the surviving cells, when administered intravenously, were identified in the injured hemisphere (Wang et al., 2004). In the present study, the HUCBC grafts probably promote neuroprotection against heatstroke-induced neuronal damage via the inhibition of iNOS/NO based on the decrease of those concentration and *N*-methyl-D-aspartate (NMDA) in brain.

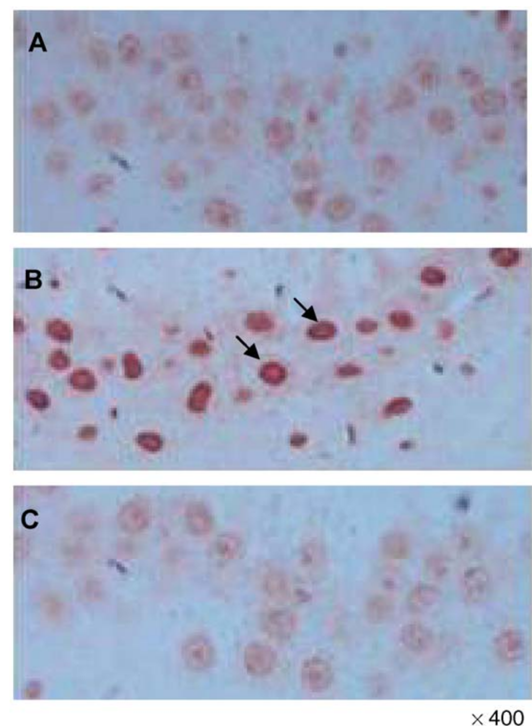

Fig. 4. Photomicrographs of iNOS staining of the striatum of a normothermic control rat (A), a PBMC (5×10^6/0.3 ml, i.v.), treated heatstroke rats (B), and a HUCBC (5×10^6/0.3 ml, i.v.)-treated heatstroke rat (C) 24 h before the initiation of heat exposure. The animals were killed at 12 min after the termination of 68-min heat exposure or the equivalent time for the normothermic controls. ×400.

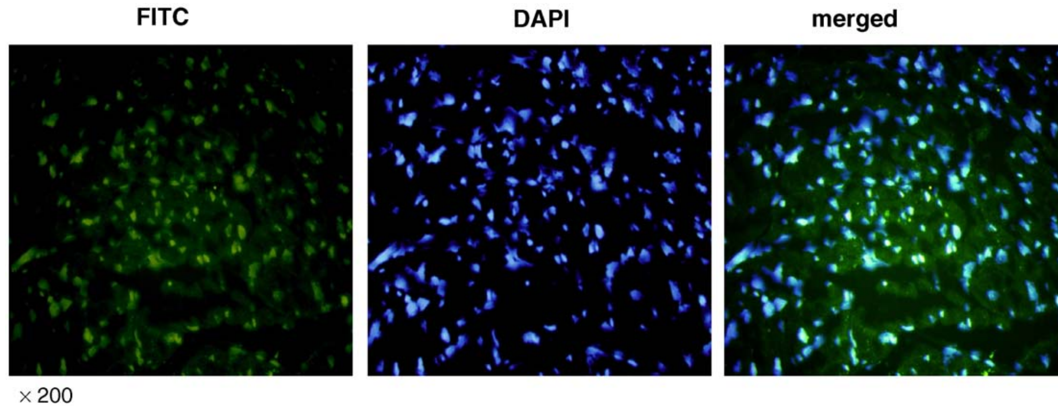

Fig. 5. HUCBC identification by human nuclei antibody immunoreactivity in the striatum of a rat that received 5×10^6 cells.

Indeed, the notion that stem cells exert an inherent neuroprotective effect was introduced by Ourednik et al. (2002) and Teng et al. (2002) and has gained great traction. Moreover, HUCBC pretreatment can maintain an appropriate level of MAP during heatstroke by reducing overproduction of cytokines as well as depression of baroreceptor sensitivity. From these results, the possible involvement of neurotrophic factors secreted by HUCBC cannot be completely eliminated. Although the surviving HUCBC were identified in different brain structures after the onset of heatstroke, the central nervous system availability of grafted cells is not a prerequisite for acute neuroprotection provided that therapeutic molecules secreted by these cells could cross the blood–brain barrier (Borlongan et al., 2004). In addition, there may be some beneficial ingredients in the HUCBC preparation, but it remains to be determined whether this must be delivered with intact cells (Wu and Kochanek, 2005).

In fact, inducible NOS under the pathological condition can be expressed in most tissues including neurons, astrocytes, and endothelial cells (Nathan and Xie, 1994). In rat brain, iNOS protein was detectable after cerebral ischemia produced by middle cerebral artery ligation (Iadecola et al., 1995) or heatstroke (Chang et al., 2004). In addition, the heatstroke-induced cerebral ischemia, iNOS-dependent NO overproduction can be suppressed by pretreatment with aminoguanidine (an inducible NOS inhibitor) (Chang et al., 2004) or HUCBC (present results). The activation of the NMDA receptors and formation of NO by iNOS may directly signal the mitochondrial release of cytochrome *C* or formation of peroxynitrite ($ONOO^-$), and subsequent hydroxyl radical production can directly damage lipids, proteins, and DNA and lead to cell death, most likely necrosis (Chan, 2001). Indeed, our previous results (Chang et al., 2003; Niu et al., 2003; Wang et al., 1997; Yang and Lin, 1999) have demonstrated that cerebral ischemia and damage during heatstroke are associated with an increased production of free radicals (specifically, hydroxyl radicals and superoxide) in the brain regions including striatum. Pretreatment with α-tocopherol, mannitol (Niu et al., 2003), magnolol (Chang et al., 2003), or heat shock preconditioning (Wang et al., 1997) significantly attenuated the heatstroke-induced cerebral ischemia and oxidative damage. Therefore, in the present results, HUCBC pretreatment may have protected against the heatstroke-induced cerebral ischemia and damage by attenuating oxidative stress in the peripheral organs (Hall et al., 2001) or in the central nervous system (Yang and Lin, 1999).

Both arterial hypotension and intracranial hypertension eventually lead to cerebral ischemia and hypoxia in rodents with heatstroke (Shih et al., 1984). The present results showed that HUCBC pretreatment protected against heatstroke by maintaining appropriate levels of MAP, CBF, and brain PO_2. The maintenance of appropriate levels of CBF and brain PO_2 in animals pretreated with HUCBC may be brought about by higher cerebral perfusion pressure resulting from lower intracranial pressure and/or higher MAP during the development of heatstroke.

Exposure of animals to hot environment induces heatstroke that is characterized by arterial hypotension, overproduction of cytokines, cerebral ischemia (Lin, 1999; Lin and Chang, 2004), and reduced baroreceptor reflex response (Li et al., 2001). It has

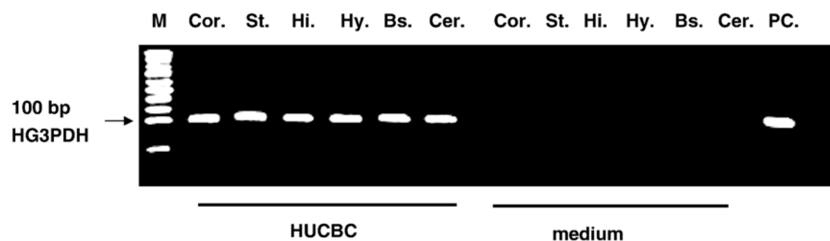

Fig. 6. Representative PCR analysis results for HG3PDH of animal injected intravenously with HUCBC (5×10^6 in 0.3 ml serum-free lymphocyte medium; left panel) or serum-free lymphocyte medium (0.3 ml). Positive control (PC) = 60 pg of human DNA extracted from cord blood. Cor., cortex; St., striatum; Hi, hippocampus; Hy, hypothalamus; Bs, brain stem; Cer., cerebellum.

been shown that an endotoxin given intravenously can elicit an increase of iNOS-dependent NO production in the nucleus tractus solitarii (NTS) and induce arterial hypotension (Lin et al., 1999). The arterial hypotension exhibited during heatstroke can be suppressed by pretreatment with an interleukin-1β receptor antagonist (Lin et al., 1995). Thus, it is likely that HUCBC pretreatment maintains appropriate levels of MAP during heatstroke by reducing overproduction of cytokines as well as depression of baroreceptor sensitivity. Of course, this needs further verification in future studies.

Finally, it should be stressed that it is still somewhat difficult to envision whether a cellular pretreatment therapy would have practical application except the most extreme and risky circumstances. Clinical application for this indication may be considered until mechanisms and parameters for beneficial outcomes are better defined. An exploration of some of the mechanisms potentially underlying the apparent protective effects of HUCBCs is explored by Vendrame et al. (2006) and Newman et al. (2006, this issue).

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