Human Umbilical Cord Blood Cells Protect Against Hypothalamic Apoptosis and Systemic Inflammation Response During Heatstroke in Rats

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Background: Intravenous administration of human umbilical cord blood cells (HUCBC) has been shown to improve heatstroke by reducing arterial hypotension as well as cerebral ischemia and damage in a rat model. To extend these findings, we assessed both hypothalamic neuronal apoptosis and systemic inflammatory responses in the presence of HUCBCs or vehicle medium immediately after initiation of heatstroke.

Methods: Anesthetized rats, immediately after the initiation of heat stress, were divided into two groups and given either serum-free lymphocyte medium (0.3 mL per rat, intravenously) or HUCBCs (5 × 10⁶ in 0.3 mL serum-free lymphocyte medium, intravenously). Another group of rats were exposed to room temperature (26ºC) and used as normothermic controls. Heatstroke was induced by exposing the anesthetized rats to a high ambient temperature of 43ºC for 68 minutes.

Results: After the onset of heatstroke, animals treated with serum-free lymphocyte medium displayed hyperthermia, hypotension, bradycardia, hypothalamic neuronal apoptosis and degeneration, and up-regulation of systemic inflammatory response molecules including serum tumor necrosis factor-alpha, soluble intercellular adhesion molecule-1 and E-selectin. Heatstroke-induced hypotension, bradycardia, hypothalamic neuronal apoptosis and degeneration, and increased systemic inflammatory response molecules were significantly inhibited by HUCBC treatment. Although heatstroke-induced hyperthermia was not affected by HUCBC treatment, the serum levels of the anti-inflammatory cytokine interleukin-10 were significantly increased by HUCBC therapy during hyperthermia.

Conclusions: These findings suggest that HUCBC transplantation may prevent the occurrence of heatstroke by reducing hypothalamic neuronal damage and the systemic inflammatory responses.
1. Introduction

Based on the understanding of the pathophysiology of heatstroke, it has been proposed that heatstroke is a form of excessive hyperthermia (core temperature rising above 40°C) associated with a systemic inflammatory response that leads to multi-organ dysfunction in which central nervous system disorders predominate.1

It is generally believed that the anterior hypothalamus preoptic area is an essential thermoregulatory center in the brain. A recent report has demonstrated that heatstroke rats display increased levels of markers for cellular ischemia (e.g. glutamate, lactate-to-pyruvate ratio and nitrite) and damage (e.g. glycerol), and enhanced expression of inducible nitric oxide synthase in the hypothalamus.2 In addition, various serum molecules including tumor necrosis factor-alpha (TNF-α),3 soluble intercellular adhesion molecule-1 (sICAM-1),4,5 and E-selectin6−8 have been demonstrated to be involved in the pathophysiology of systemic inflammatory response syndrome.9−11 Hyperthermia, which occurs during heatstroke, may facilitate the leakage of endotoxin from the intestine to the systemic circulation and result in excessive activation of leukocytes and endothelial cells.1 Thus, heatstroke is characterized by the release of TNF-α and other cytokines, up-regulation of cell-surface adhesion molecules, and release of soluble cell-surface adhesion molecules (e.g. E-selectin and ICAM-1).

Human umbilical cord blood cells (HUCBCs) have emerged as an alternative to bone marrow because of their greater availability, lower risk of mediating viral transmission, and weaker immunogenicity.12 Evidence has accumulated to show 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.13−16 We have demonstrated that HUCBC therapy may resuscitate rats with heatstroke by reducing circulatory shock and cerebral ischemia.17 However, it is unknown whether the hypothalamic neuronal damage and the systemic inflammatory responses that occur during heatstroke can be reduced by HUCBC administration.

This study possessed two objectives. The first was to assess whether neuronal degeneration and apoptosis in the hypothalamus, both of which occur during heatstroke, could be reduced by HUCBC administration. The second was to assess whether heatstroke-induced up-regulation of TNF-α, E-selectin and ICAM-1 in the peripheral blood stream could be ameliorated by HUCBC administration.

2. Materials and Methods

2.1. Animals

Adult Sprague-Dawley rats (weighing 294±15g) were obtained from the Animal Resource Center of the National Science Council of the Republic of China (Taipei, Taiwan). The animals were housed four per group at an ambient temperature (Ta) of 22±1ºC, with a 12-hour 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, and 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 hours in duration) 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.

2.2. Surgery and physiological parameter monitoring

The right femoral artery and vein of rats were cannulated with polyethylene tubing (PE50), under urethane anesthesia, for blood pressure monitoring and drug administration. The core temperature (Tco) was monitored continuously by a thermocouple, while mean arterial pressure (MAP) and heart rate (HR) were monitored continuously with a pressure transducer.

2.3. Induction of heatstroke

The Tco of the anesthetized animals were maintained at about 36°C with an infrared light lamp, except during the heat stress experiments. Heatstroke was induced by placing the animals in a folded heating pad maintained at 43°C by circulating hot water. The time at which the MAP decreased irreversibly from the peak was taken as the onset of heatstroke.18 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 68±2 minutes (n=8). Therefore, in the following groups of heatstroke rats, all animals were exposed to 43°C for exactly 68 minutes and were then allowed to recover at room temperature (26°C).
2.4. Experimental groups

Animals were assigned randomly to one of three groups. One group of rats, treated with an intravenous dose of vehicle solution (0.3 mL serum-free lymphocyte medium per rat) was exposed to a Ta of 26°C, and the physiological parameters were continuously recorded for up to 480 minutes (or to the end of the experiments). This group of rats was used as normothermic controls. The second group was treated with the same dose of vehicle solution after the initiation of heat exposure (Ta 43°C for 68 minutes) and was used as vehicle-treated heatstroke animals. The third group of rats was treated with an intravenous dose of HUCBC (5 × 10^6 in 0.3 mL serum-free lymphocyte medium) immediately after the initiation of heat exposure (Ta 43°C for 68 minutes). The last two groups of rats were exposed to heat exposure (43°C) for exactly 68 minutes to induce heatstroke and were then allowed to recover at room temperature (26°C). Physiological parameters and survival time (interval between the initiation of heat exposure and animal death) were observed for up to 480 minutes (or to the end of the experiments).

2.5. Preparation of HUCBCs

Human HUCBCs were obtained from the freshly collected buffy coat fraction from healthy donors at the Chi Mei Medical Center (Tainan, Taiwan). This project was approved by the Institutional Review Board of Chi Mei Medical Center. HUCBCs were isolated by centrifugation over a Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient at 400×g for 30 minutes at room temperature in a Sowall RT600 B (Du Pont, CO., Wilmington, DE, USA). The cells collected at the interface were washed three times in serum-free Roswell Park Memorial Institute-1640 (BRL, Grand Island, NY, USA) and subsequently resuspended in serum-free lymphocyte medium (Gibco, BRL). For intravenous administration, a 26-gauge needle was inserted into the rat’s tail vein, and cells (0.3 mL) were delivered over a 1-minute period.

2.6. Neuronal damage score and apoptosis

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 hours prior to removal from the skull. The brain was removed and embedded in paraffin blocks. Serial sections (10 μm thick) through the hypothalamus were stained with hematoxylin and eosin for microscopic evaluation. The extent of neuronal damage was scored on a scale of 0–3, modified from the grading system of Pulsinelli et al., 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 by an examiner blinded to the experimental conditions.

The TUNEL assay was performed using the same hypothalamic tissue used in histological verification. Color was developed using 3,3′-diaminobenzidine tetrachloride (Sigma Chemical Co., St. Louis, MO, USA). Sections were treated with xylene and ethanol to remove paraffin and for dehydration. They were then washed with phosphate buffered saline (PBS) and incubated in 3% hydrogen peroxide solution for 20 minutes. The sections were treated with 5 μg/mL proteinase k for 2 minutes at room temperature, and rewarshed in PBS (0.1M, pH 7.4). The sections were then treated with a TUNEL reaction mixture (terminal deoxynucleotidyl transferase, nucleotide mixture, Roche, Mannheim, Germany) at 37°C for 1 hour, and the sections were washed with distilled water. They were then incubated in anti-fluorescein, check antibody-conjugated with horseradish peroxidase at room temperature for 30 minutes, washed and visualized using the avidin-biotin complex (ABC) technique and 0.05% 3,3′-diaminobenzidine tetrachloride as a chromogen. The numbers of TUNEL-positive cells were counted by a pathologist at 200× magnification, 30 fields per section. Blinding was performed for the pathologist’s grading of results.

2.7. Measurement of serum TNF-α, ICAM-1 and IL-10 levels

Blood samples were collected, immediately separated, and stored at −80°C until they could be assayed. We used commercially available ELISA kits for the determination of serum TNF-α, ICAM-1 and IL-10 levels (Quantikine, R&D Systems Inc. Minneapolis, MN, USA) according to the manufacturer’s instructions.

2.8. Measurement of E-selectin

Rat peripheral polymorphonuclear (PMN) cells were isolated from the whole blood of rats and treated with heparin (100 units/mL). Erythrocytes were allowed to sediment for 30 minutes after the addition of 3 mL of 6% dextran (weight/volume in PBS) to 10 mL blood. After sedimentation, the plasma containing leukocytes was centrifuged twice at 300g for 5 minutes each. The precipitates were mixed with 70% osmolality-adjusted Percoll and centrifuged at 30,000g for 30 minutes at 26°C.
The PMN-rich layer was fractionated. Each fraction was washed twice with Hanks’ balanced salt solution, and the cell number was counted. The purity of the PMNs was determined to exceed 95% by Giemsa staining. Cells (1 × 10^6 cells/tube) were incubated with a rabbit polyclonal antibody to CD62E (ab18981; Abcam PIC332 Cambridge, UK) or control. After washing, the cells were stained with a secondary antibody (goat polyclonal to rabbit IgG-H&L [FITC] ab6717; Abcam PIC). Cells were incubated for 1 hour at 4°C and washed. The cells were mixed with oligosaccharides and incubated for 20 minutes, and then co-incubated with KM93 for 60 minutes. The fluorescence intensity of cells was analyzed with a FACStar (Becton Dickinson).

2.9. Statistical analysis

All data are expressed as means ± standard deviation. One-way analysis of variance with Tukey’s multiple comparisons test was used for serum markers. The Wilcoxon test was used for histological assessment. Significant differences were established at p < 0.05. For all statistical analyses, SPSS software version 10.0 (SPSS Inc., Chicago, IL, USA) was used.

3. Results

3.1. HUCBC treatment attenuates hypotension and improves survival during heatstroke

Table 1 summarizes the survival time for vehicle-treated and HUCBC-treated rats during heatstroke.

![Table 1](image)

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Survival time (min)</th>
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<tbody>
<tr>
<td>Normothermic controls</td>
<td>&gt;480 (n=8)</td>
</tr>
<tr>
<td>Vehicle-treated heatstroke rats*</td>
<td>22±2 (n=8)†</td>
</tr>
<tr>
<td>HUCBC-treated heatstroke rats*</td>
<td>214±23 (n=8)‡</td>
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Except for the normothermic controls, data are means ± standard deviation followed by the number of animals used in parentheses. Group 1 was terminated about 480 minutes after the initiation of heat exposure. *For both groups exposed to 43°C, the heat stress was withdrawn at 68 minutes and the rats were allowed to recover at room temperature (26°C). †p<0.05 compared with normothermic controls; ‡p<0.05 compared with vehicle-treated heatstroke rats; HUCBC = human umbilical cord blood cells.

The survival time values were 20–24 minutes (n=8) and 209–237 minutes (n=8) for vehicle-treated and HUCBC-treated rats, respectively. The HUCBC-treated heatstroke rats had significantly longer survival time compared with vehicle-treated heatstroke rats (p<0.05).

Figure 1 shows the effect of heat exposure (43°C for 68 minutes) on Tco, MAP and HR in rats treated with vehicle medium and in rats treated with HUCBCs. As shown in this figure, 22 minutes after the termination of heat exposure in the vehicle-treated group, the values of both MAP and HR were significantly lower than those of normothermic controls (p<0.05). On the other hand, the values for Tco in the vehicle-treated heatstroke rats were significantly higher than those of the normothermic controls. Heatstroke-induced hypotension and bradycardia, but not hyperthermia, were significantly reduced by HUCBC treatment.

3.2. HUCBC treatment attenuates hypothalamic apoptosis and neuronal degeneration during heatstroke

Figure 2 summarizes the effects of heat exposure on the number of TUNEL-positive cells in the hypothalamus of normothermic controls, vehicle-treated heatstroke rats, and HUCBC-treated rats. At 22 minutes after the onset of heatstroke, the number of TUNEL-positive cells of the hypothalamus was greater in vehicle-treated heatstroke rats than in the normothermic controls. However, increase of TUNEL-positive cells in the hypothalamus of heatstroke rats was greatly attenuated by HUCBC. A typical example of TUNEL staining of the hypothalamus is shown in the top panel of Figure 2.

As shown in Table 2, after the onset of heatstroke, the hypothalamic neuronal damage scores were higher in animals treated with vehicle compared with the normothermic controls. Histopathological verification revealed that heatstroke caused cell body shrinkage, pyknosis of the nucleus, loss of Nissl substance, and disappearance of the nucleus in the hypothalamus of vehicle-treated rats (Figure 3). However, HUCBC treatment had neuroprotective effects (Figure 3).

3.3. HUCBC treatment up-regulates serum IL-10 levels but down-regulates serum E-selectin, ICAM-1 and TNF-α levels during heatstroke

Figure 4 shows the serum levels of E-selectin, ICAM-1, TNF-α and IL-10 among the three experimental groups. Compared with the normothermic controls, vehicle-treated heatstroke rats had higher levels of E-selectin, ICAM-1 and TNF-α at 22
minutes after the onset of heatstroke. The increase in the serum levels of these three markers caused by heatstroke were significantly reduced by HUCBC therapy. However, compared with the vehicle-treated rats, HUCBC-treated rats had higher serum levels of IL-10 at 22 minutes after the onset of heatstroke.

4. Discussion

Heatstroke is defined as a condition in which the core temperature is elevated to a critical level that induces multi-organ damage and dysfunction.20–22 The severity of illness depends on the degree of hyperthermia and its duration.23 The current approach for treatment of heatstroke is whole-body cooling.1 However, heatstroke is often fatal following adequate body cooling.24,25 Tissue damage continues to develop despite cooling of the whole body to the normal body temperature in 25% of heatstroke patients.26 It has also been shown that normal volunteers can passively endure a core temperature of about 42ºC with none or minimal tissue injury.27,28 Indeed, as demonstrated in the present study, in the absence of whole-body cooling, HUCBC treatment significantly prevented the occurrence of heatstroke syndromes without affecting the induced hyperthermia. The same contention has been previously proposed.29 Thus, it appears that tissue ischemia and hypoxia, rather than hyperthermia, are the main causes of heatstroke.

Evidence has accumulated to suggest that thermoregulatory deficits may occur during heatstroke. For example, unanesthetized, unrestrained heatstroke mice displayed hypothermia when exposed to room temperature.2,30–32 The hypothermia that occurred after onset of heatstroke may have resulted from neuronal apoptosis and cell degeneration in the hypothalamus (as demonstrated in the present study). In the current study, we also demonstrated the protective effects of HUCBCs in reducing heatstroke-induced hypothalamic neuronal apoptosis and degeneration.

Figure 1  Effects of heat stress (ambient temperature [Ta] 43°C for 68 minutes) on core temperature, mean arterial pressure and heart rate. Open circles, values at Ta of 43°C in eight rats treated with vehicle immediately after the onset of heat exposure. Solid circles, values at Ta of 43°C in eight rats treated with human umbilical cord blood cells (5×10⁶/0.3 mL, intravenously) immediately after the initiation of heat exposure. Another eight rats were used as normothermic controls (solid triangles). Values are means±standard deviation. *p<0.05 compared with the normothermic controls; † p<0.05 compared with the vehicle-treated group (at 43°C). HR=heart rate; MAP=mean arterial pressure; Ta=ambient temperature; Tco=core temperature.
HUCBCs protect heatstroke rats

Figure 2  Histological examination of neuronal damage. The photomicrographs of the hypothalamus in a normothermic control rat (A), a heatstroke rat treated with vehicle (B), or a heatstroke rat treated with human umbilical cord blood cells (5 x 10^6/0.3 mL, intravenously) (C) immediately after the initiation of heat stress. Twenty-two minutes after 68-minute heat exposure, the hypothalamus of the rat treated with vehicle showed cell body shrinkage, pyknosis of the nucleus, loss of Nissl substance, and disappearance of the nucleolus (B). By contrast, administration of human umbilical cord blood cells reduced neuronal damage, as shown in panel C. Magnification x 400.

Table 2  Effects of heat exposure (43°C for 68 minutes) on neuronal damage score values of the hypothalamus in rats treated with vehicle medium or human umbilical cord blood cells (5 x 10^6/0.3 mL, intravenously) immediately after the initiation of heat exposure

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Neuronal damage score (0–3)</th>
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<tbody>
<tr>
<td>Normothermic controls</td>
<td>(0, 0.75)</td>
</tr>
<tr>
<td>Vehicle-treated heatstroke rats</td>
<td>2 (2, 2)*</td>
</tr>
<tr>
<td>HUCBC-treated heatstroke rats</td>
<td>1 (0.25, 0.75)†</td>
</tr>
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</table>

Values are medians with the first and third quartile in parentheses for eight rats per group. To determine neuronal damage score, the animals were killed after 68 minutes of heat exposure.

HUCBCs are transplanted into the neonatal subventricular zone at least some of them differentiate into neuronal and glial phenotypes within this neurogenic region. Upon administration of HUCBCs after stroke, traumatic brain injury and heatstroke, there were significant improvements in behavioral indicators or survival. After treatment with HUCBCs, the neuronal damage that occurred during heatstroke was greatly reduced. Future studies are required to ascertain whether heatstroke-induced hypothalamic neuronal damage can be repaired by HUCBC therapy.

The plasma levels of inflammatory cytokines such as TNF-α and interleukin-1β are elevated in persons with heatstroke. Studies in rats and rabbits have also shown that heatstroke induces systemic and brain production of TNF-α and interleukin-1β. The increase in the plasma levels of these proinflammatory cytokines is associated with the severity of heatstroke. Evidence has also suggested that IL-10 may have a therapeutic potential in acute and chronic inflammatory disease. For example, IL-10-knockout mice have an increased likelihood of inflammatory illness and higher mortality rates.
Figure 3  TUNEL-positive cells. *The number of TUNEL-positive cells in the hypothalamic sections 22 minutes after the termination of heat stress was significantly \((p<0.05; n=8)\) increased in vehicle-treated rats compared with normothermic controls. †The number of TUNEL-positive cells in the hypothalamic sections 22 minutes after the termination of heat stress was significantly \((p<0.05; n=8)\) decreased in heatstroke rats treated with human umbilical cord blood cells \((5 \times 10^6/0.3 \text{ mL, intravenously})\) compared with vehicle-treated rats. HS=heatstroke; HUCBC=human umbilical cord blood cells; NC=normothermic controls.

Figure 4  Serum levels of tumor necrosis factor-alpha (TNF-\(\alpha\)), intercellular adhesion molecule (ICAM), E-selectin and interleukin (IL)-10 among the three experimental groups. *Serum levels of TNF-\(\alpha\), IL-10, E-selectin and ICAM-1 determined 22 minutes after the termination of heat stress were significantly \((p<0.05; n=8)\) increased in vehicle-treated rats compared with normothermic controls. †Serum levels of TNF-\(\alpha\), E-selectin and ICAM-1 determined 22 minutes after the termination of heat stress were significantly \((p<0.05; n=8)\) decreased and the serum levels of IL-10 were significantly \((p<0.05; n=8)\) increased in heatstroke rats treated with human umbilical cord blood cells \((5 \times 10^6/0.3 \text{ mL, intravenously})\) compared with vehicle controls. HS=heatstroke; HUCBC=human umbilical cord blood cells; ICAM=intercellular adhesion molecule; IL=interleukin; NC=normothermic controls; TNF-\(\alpha\)=tumor necrosis factor-alpha.
after experimental sepsis. Exogenous administration of recombinant IL-10 protects mice from lethal endotoxemia by reducing TNF- release. In endotoxic mice, neutralization of endogenously produced IL-10 results in an increased production of proinflammatory cytokines and enhanced mortality.

In the present study, we showed that administration of HUCBC increased the serum levels of IL-10 and decreased the levels of TNF-α, and prolonged the survival time during heatstroke. In addition, glucocorticoids, interleukin-1 receptor antagonists and TNF-α may restore tissue blood flow and homeostatic function and limit multi-organ dysfunction and death in heatstroke. In terms of survival time, our previous studies have shown that cortisone and an IL-1 receptor antagonist have similar potency to HUCBCs in treating heatstroke. Therefore, we need to evaluate the therapeutic effects of the combination of HUCBCs and anti-inflammatory or anti-cytokine drugs during heatstroke in future studies.

TNF-α plays critical roles as a mediator of inflammatory responses. The expression of adhesion molecules is also induced by TNF-α, resulting in increased leukocyte-endothelial adherence and activation of leukocytes. Activation of leukocyte adhesion to the endothelium and migration of leukocytes into the tissue where cytotoxic chemicals are released causes tissue injury. The adhesion molecule ICAM-1 mediates firm adhesion between leukocytes and endothelial cells and contributes to the migration of leukocytes from post-capillary venous into the reperfused tissue. During inflammation, endothelial cells express ICAM-1, which initiates adhesion and transendothelial migration of circulating leukocytes. The serum TNF-α and ICAM-1 levels can be considered as markers for the systemic inflammatory response because they indirectly reflect the whole-body production of TNF-α and ICAM-1 in various organs.

CD62E (E-selectin) is an endothelial cell-specific selectin that is expressed on cytokine-induced endothelial cells only after activation by proinflammatory cytokines. E-selectin has been associated with the blood vessel endothelium in diverse inflammatory situations. Yoshida et al reported that E-selectin expressed in endothelial cells becomes associated with the actin cytoskeleton during leukocyte adhesion, suggesting that E-selectin engages in transmembrane signaling upon ligand binding. The serum levels of TNF-α, E-selectin and ICAM-1 were upregulated in patients with heatstroke. The present results further demonstrated that the increased serum levels of these molecules during heatstroke in a rat model could be significantly reduced by HUCBC treatment. Considering these observations together, it is apparent that the heatstroke-induced systemic inflammatory response can be attenuated by HUCBC therapy.

5. Conclusions

In summary, our results demonstrate that, in the absence of whole-body cooling, HUCBC treatment significantly prevents the occurrence of heatstroke syndrome without affecting the induced hyperthermia. Heatstroke-induced hypothalamic neuronal apoptosis and degeneration, and the systemic inflammatory response (as demonstrated by increased serum levels of TNF-α, E-selectin and ICAM-1) can be significantly prevented by HUCBC treatment. These findings indicate that HUCBC transplantation may improve heat tolerance by reducing the occurrence of hypothalamic neuronal apoptosis and degeneration, and the systemic inflammatory response.

Acknowledgments

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