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BRIEF REPORT

Antiepileptic and neuroprotective effects of human umbilical cord blood mononuclear cells in a pilocarpine-induced epilepsy model

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Abstract Status epilepticus (SE) is a condition of persistent seizure that leads to brain damage and, frequently, to the establishment of chronic epilepsy. Cord blood is an important source of adult stem cells for the treatment of neurological disorders. The present study aimed to evaluate the effects of human umbilical cord blood mononuclear cells (HUCBC) transplanted into rats after induction of SE by the administration of lithium and pilocarpine chloride. Transplantation of HUCBC into epileptic rats protected against neuronal loss in the hippocampal subfields CA1, CA3 and in the hilus of the dentate gyrus, up to 300 days after SE induction. Moreover, transplanted rats had reduced frequency and duration of spontaneous recurrent seizures (SRS) 15, 120 and 300 days after the SE. Our study shows that HUCBC provide prominent antiepileptic and neuroprotective effects in the experimental model of epilepsy and reinforces that early interventions can protect the brain against the establishment of epilepsy.

Keywords Epilepsy · Pilocarpine · Cord blood · Cell therapy · Neuroprotection · Seizure frequency · Neuronal loss

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Introduction

The experimental model of temporal lobe epilepsy (TLE), induced in rats by administration of lithium and pilocarpine, resembles many aspects of human TLE. Beginning with the occurrence of a brain injury with damage to neuronal subfields of the hippocampus, status epilepticus (SE), followed by a latent phase called epileptogenesis and by the establishment of spontaneous recurrent seizures (SRS). There is currently great interest on the development of therapeutic strategies capable of modulating the process of epileptogenesis (Löscher and Brandt 2010), since there is currently no clinically approved therapy to modulate this process. The lithium–pilocarpine model of TLE is commonly used to investigate the anticonvulsant effects of antiepileptic drugs and to study mechanisms involved in epileptogenesis and its progression to the chronic phase of epilepsy (Turski et al. 1989).

Stem cells are candidate for therapeutic use in epilepsy, not only because of their ability of differentiation and fusion with resident cells, but also due to the secretion of soluble factors that could interfere in the process of epileptogenesis by inducing neuroprotection and preventing formation of abnormal circuitry, as previously shown (Costa-Ferro et al. 2010, 2012; Venturin et al. 2011). Stem cells have been isolated from various tissues in animals and humans. Cord blood (Lu et al. 1996), which is a rich source of mesenchymal stem cells (Erices et al. 2000) and endothelial precursors (Nieda et al. 1997). Human umbilical cord blood mononuclear cells (HUCBC) have been previously shown to possess neuroprotective effects and to induce functional, behavioral and morphological improvements on experimental models of cerebral ischemia (Arien-Zakay et al. 2011), traumatic brain injury (Newcomb et al. 2006), spinal cord injury (Park et al. 2011) and amyotrophic lateral sclerosis (Garbuzova-Davis et al. 2003). However, at the time of writing, there is no data about the effects of HUCBC transplantation in experimental epilepsy.

In this study we investigated the effects of HUCBC transplantation on the frequency and duration of SRS, as well as on the extent of neuronal damage in the hippocampus as a result of SE in the Lithium–pilocarpine model of TLE.

Materials and methods

All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the FIOCRUZ-BA. Figure 1a shows a schematic of the study design. Male Wistar rats 50–60 days old (n = 120) were injected with lithium chloride (3 mequiv./kg i.p.; Vetec Química Fina, Duque de Caxias, Brazil) 18–20 h before seizure induction. On the following day, a group of 21 rats (non-SE) was randomly selected to receive saline injection, while the remaining 108 rats were injected with methylscopolamine (1 mg/kg i.p., Sigma–Aldrich; St. Louis, MO, USA) to reduce the peripheral cholinergic effects of pilocarpine, followed 30 min later by pilocarpine hydrochloride (50 mg/kg i.p.; Sigma–Aldrich). The Racine scale for the behavioral assessment of seizure was used to evaluate the progression to SE (Racine 1972) and the animals were considered to have progressed on to SE when stage V was observed. Diazepam (10 mg/kg i.p.) was administered 90 min after the onset of SE, to block seizures. All rats administered with lithium–pilocarpine that developed SE (grade V in Racine's Scale) were included in this study. The animals that survived SE (n = 55) were divided randomly into two groups: (1) lithium–pilocarpine injected with saline (SE-Saline: n = 28) (2) lithium–pilocarpine transplanted with HUCBC (SE-HUCBC; n = 27).

Umbilical cord blood samples were donated from healthy donors at CordCell-Umbilical Cord Blood Stem Cell Center (São Paulo, Brazil) and the donors' parents signed free informed consent forms. HUCBC were isolated by centrifugation over a Ficoll-Hipaque (Pharmacia, Uppsala, Sweden) density gradient. The cells were counted, labeled with CellTracker^{TM} Green CMFDA, according to manufacturer's instructions (Molecular Probes; Carlsbad, CA, USA). This is a dye that contains a chloromethyl group, being nonfluorescent until activation by cleavage by intracellular esterases, when it is transformed into a cell-impermeant reaction product, endowed with green fluorescence (Fig. 1). After labeling, the cells were administered through the tail vain (1×10^6 cells/rat), right after diazepam injection. Equal volume of cell-free saline was injected into the SE-saline rats. In addition, samples of unlabeled HUCBC were stained with the following conjugated antibodies: CD45-APC, CD31-FITC, CD34-PerCP-CY5.5, CD90-FITC, CD105-FITC, CD133-PE, CD117-PE (all purchased from BD Biosciences; San Diego, CA), and analyzed by flow cytometry in a LRSII flow cytometer (BD Biosciences).

The rats were videorecorded 15, 120 and 300 days after SE. The recordings were performed for 14 h a day (7 h during the light period/7 h during the dark period) during 14 days. Seizures were scored according to the Racine's standard scoring system (Racine 1972). All of the rats were euthanized after the last recording period. Serial sections of the hippocampus, with 6-μm thickness and 150-μm intervals, were obtained in the levels corresponding to bregma −2.8 mm to −5.6 mm of Paxinos and Watson (1996). Cell counts were performed on CA1, CA3a and b, and hilus regions of the hippocampus. Nine equidistant sections were obtained from each animal and stained with Nissl stain (Cresyl fast violet). The images were obtained with a 400× magnification, and neurons were

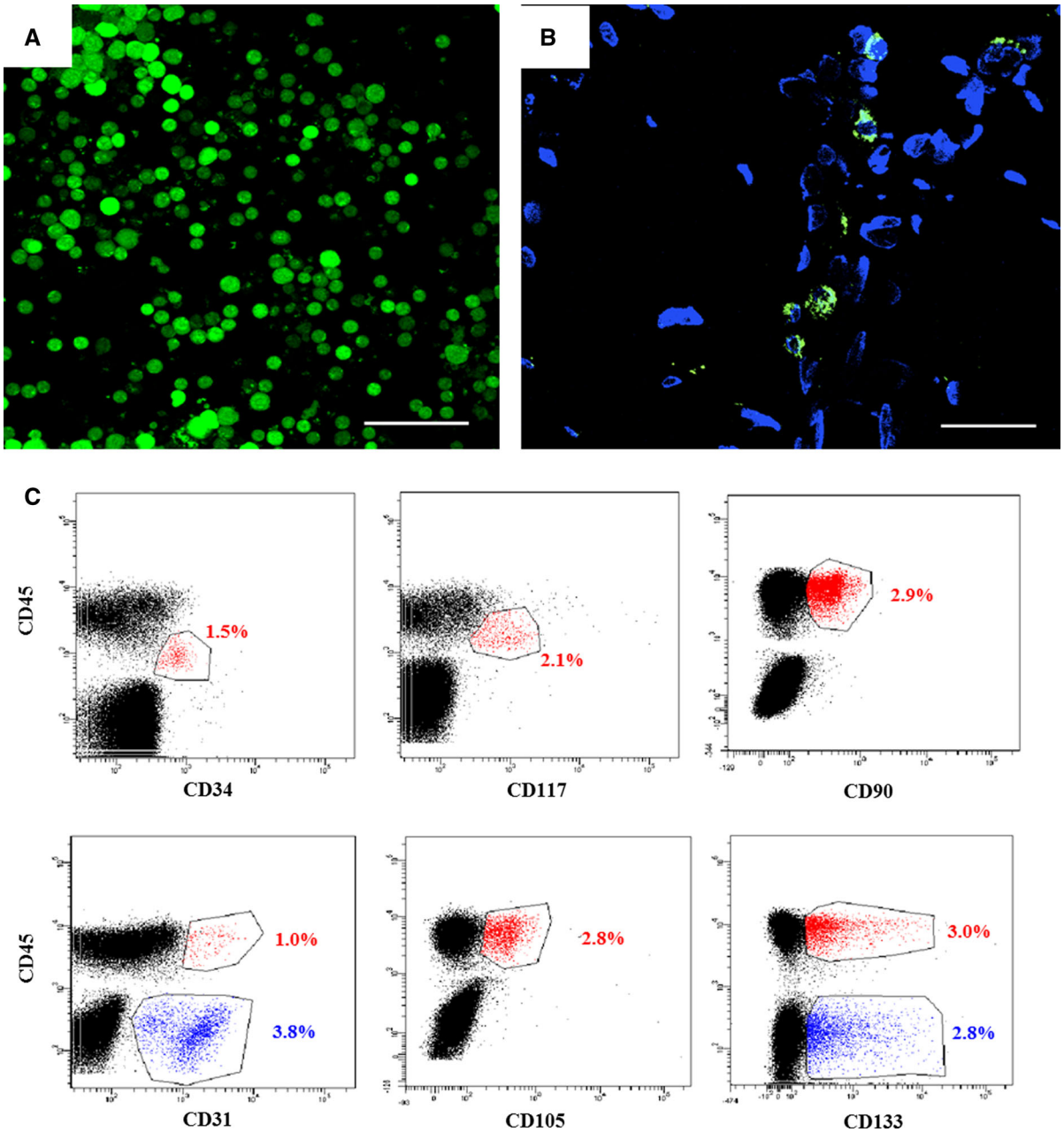

Fig. 1 Transplantation of HUCBC into epileptic rats. **a** HUCBC stained with cell tracker green before transplantation. **b** HUCBC (green) found in the choroid plexus of rats 2 days after the transplantation. Nuclei were stained with DAPI (blue). *Scale bar* represents 50 μm. **c** Flow cytometry analysis of HUCBC stained with anti CD45, CD34, CD90, CD117, CD31, CD105 and CD133. (Color figure online)

counted within a frame with an area of 1.600 μm^2, using the Image-Pro Plus analysis software version 7.0 (Media Cybernetics, Inc.; Bethesda, MD, USA). Neuronal densities were calculated and corrected by Abercrombie's formula (Abercrombie 1946).

The presence of donor cells in the brains of transplanted rats was evaluated by fluorescence microscopy 48 h after transplantation in the brains of four randomly selected rats. 10 μm-thick brain sections obtained from frozen samples and stained with

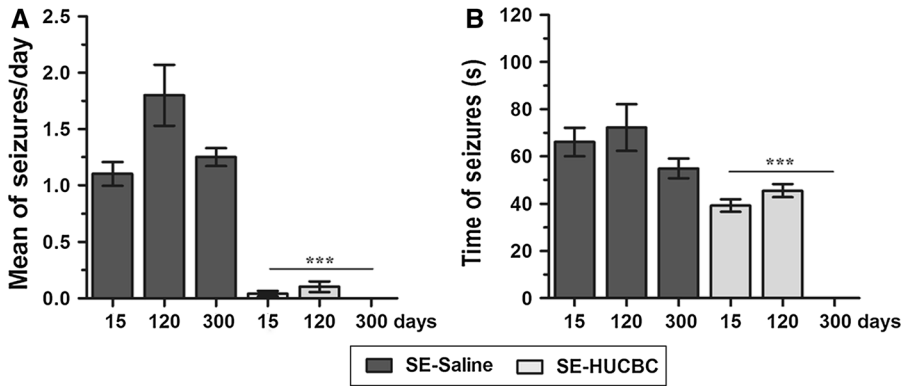

Fig. 2 Recurrent spontaneous seizures recorded 15, 120 and 300 days after SE in rats that received saline solution (SE-saline) or HUCBC (SE-HUCBC). **a** frequency and **b** duration of seizures. Results are expressed as the mean ± SEM of 28 (SE-Saline) and 23 (SE-HUCBC) rats, respectively. ***$p < 0.001$

DAPI (Vector; Burlingame, CA, USA). For each rat, four brain sections, obtained from bregma −2.8 mm to −5.6 mm, were analyzed. For microscopy analysis, an Olympus FluoView 1000 confocal laser-scanning microscope (Olympus; Tokyo, Japan) was used for image acquisition. Cells stained with CellTracker™ Green CMFDA were identified in the brain as green fluorescent cells.

The latency to seizure onset, seizure duration, seizure frequency and neuronal loss were analyzed by ANOVA followed by Tukey's test. All analyses were made using the Prism statistical software version 5.01 (GraphPad Software; San Diego, CA, USA) and a $p < 0.05$ was considered statistically significant.

Results

The immunophenotype of HUCBC was CD45 (43.2 %), CD34 (1.5 %), CD117 (2.1 %), CD90 (1.5 %), $CD45^{-}CD31^{+}$ (3.8 %), $CD45^{-}CD105^{+}$ (0.1 %) and $CD45^{-}133^{+}$ (2.8 %) (Fig. 1). After injection of pilocarpine, a mean latency time of 21.24 ± 7.17 min was observed before the onset of SE. Mortality rates did not differ between saline-injected and HUCBC-transplanted groups. Two days after the SE, we found cell-tracker^{+} cells in the brains of transplanted rats in about 0.02 % of the fields analyzed (Fig. 1b).

Fifteen days after SE, a frequency of 1.10 ± 0.10 seizures/day was observed in saline-treated versus 0.04 ± 0.02 seizures/day in HUCBC-treated rats (Fig. 2a). At the second time analyzed-120 day after SE—a frequency of seizures increased to 1.80 ± 0.27 seizures/day in saline-treated versus 0.10 ± 0.04 seizures/day in HUCBC treated rats. After 300 days, saline-treated rats had frequency of 1.25 ± 0.07 seizures/day, while those treated with HUCBC did not present seizures at this time point ($p < 0.001$, SE-Saline vs SE-HUCBC).

The duration of SRS was also reduced in rats treated with HUCBC when compared to that of saline-treated group (Fig. 2b). Fifteen days after SE, the mean convulsive time was 66.10 ± 6.03 s and 39.28 ± 2.58 in saline-treated and HUCBC-treated rats, respectively, whereas 120 days after SE, it was 72.22 ± 2.7 s and 45.49 ± 2.7 s in saline-treated and HUCBC-treated rats, respectively. After 300 days of SE, a mean convulsive time of 54.87 ± 4.15 was observed in saline-treated rats, while no seizures were detected in the HUCBC-treated group.

Neuronal densities in hippocampal subfields were higher in the group treated with HUCBC when compared to rats injected with saline (Fig. 3). There was a significant difference between the neuronal counts of the non-SE and SE-Saline rats, the latter showing an extensive neuronal loss (88 % cell loss in hippocampal pyramidal cell layer CA1, 81 % in CA3a, 68 % in CA3b and 62 % in the hilus of DG). SE-HUCBC rats had less neuronal damage than rats from the SE-Saline group in all subfields evaluated. The neuronal loss was approximately of 42 % in CA1, 41 % in CA3a and b, and 13 % in the hilus of DG by treatment with HUCBC ($p = 0.001$, SE-HUCBC vs SE-Saline). The neuronal damage in hippocampal subfields was observed by light microscopy analysis of the hippocampi from non-SE and SE rats (Fig. 3).

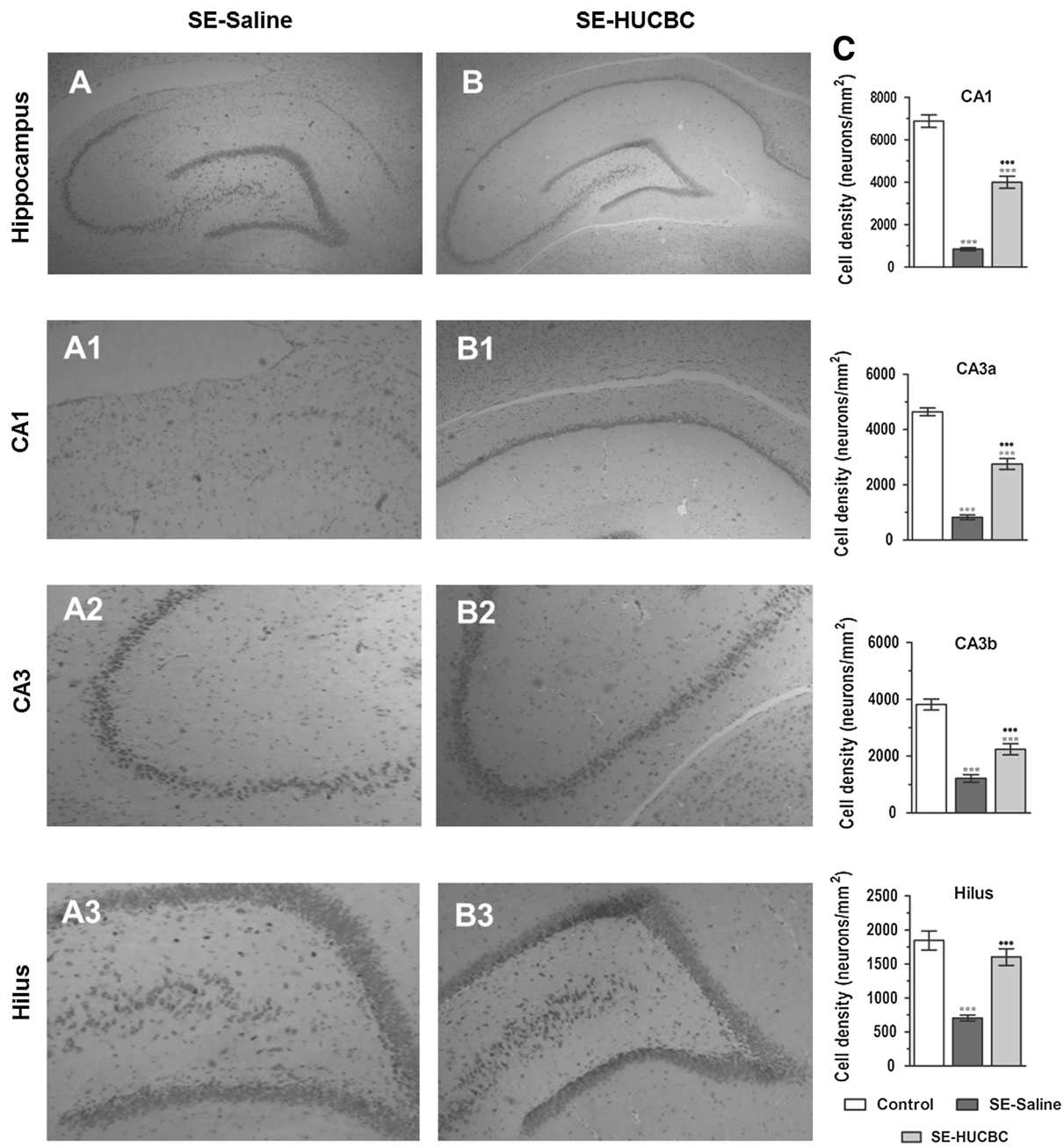

Fig. 3 Representative Nissl-stained sections of the hippocampus 300 days after SE acquired with ×40 magnification. **A** SE-Saline, demonstrating increased neuronal loss when compared to (**B**) SE-HUCBC. **A1–3** and **B1–3** are magnified views of regions from **A** and **B**. **C** Neuronal density of CA1, CA3 and hilus of GD of the hippocampus in 300 days after SE and transplantation of HUCBC. Data are presented as mean ± SEM of 5 rats per group. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{\bullet\bullet\bullet}p < 0.001$ compared to control

Discussion

Our study provides evidence that intravenous administration of HUCBC prevents development of chronic epilepsy and causes neuroprotection when given after SE, despite the small numbers of donor cells found in the brain. Similar results were previously shown for the transplantation of bone marrow mononuclear cells

(Costa-Ferro et al. 2010, 2012; Venturin et al. 2011). These observations suggest that these cells promote neuroprotection, possibly acting as a source of trophic factors and cytokines, which may increase neurogenesis, limit neuronal damage, reduce mossy fiber sprouting and the occurrence of SRS (Simonato and Zucchini 2010; Paradiso et al. 2011). There is increasing evidence that in fact cell therapy in the central nervous system induces tissue regeneration not by cell differentiation into neural cells but mainly by paracrine mechanisms (Uccelli 2013).

Increasing evidence also highlights the possible involvement of inflammatory processes arising from injured brain in the development of epilepsy (i.e., in epileptogenesis) (Vezzani and Friedman 2011). Pro-inflammatory mediators can alter neuronal excitability, favouring seizures (Vezzani et al. 2011). Giving the importance of inflammatory processes in the brain to the pathogenesis of epilepsy, it is possible that the mechanisms of action of HUCBC also involve immunomodulatory actions. Those actions were previously reported in an experimental model of stroke, in which the efficacy of HUCBC transplantation at later time points was partially attributed to their anti-inflammatory action, leading to a reduction in the number of B cells and CD11b-expressing monocytes/macrophages in the brain (Vendrame et al. 2005). In line with this idea, we recently demonstrated that transplantation of bone marrow mononuclear cells decreases the production of inflammatory cytokines in chronic epileptic rats. In this study we also demonstrated that the protective effects of bone marrow mononuclear cells were not only observed in experiments with isogenic cells, but also with xenogenic cells (Costa-Ferro et al. 2012).

In conclusion, the present study reinforces that early interventions can protect the brain against the establishment of epilepsy. Further studies exploring the molecules secreted by HUCBC may bring important mechanistic knowledge for the development of new therapies for epileptic patients.

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