

Lack of migration and neurological benefits after infusion of umbilical cord blood cells in ischemic brain injury

Malgorzata Zawadzka¹, Katarzyna Lukasiuk¹, Eugeniusz K. Machaj^{2,3}, Zygmunt Pojda^{2,3},
and Bozena Kaminska^{1*}

¹Laboratory of Transcription Regulation, The Nencki Institute of Experimental Biology, Warsaw, Poland,

*Email: bozenakk@nencki.gov.pl; ²Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland; ³Institute of Hygiene and Epidemiology, Warsaw, Poland

The finding of stem/progenitor cells in postnatal bone marrow and umbilical cord blood, opens up a possibility of using stem cells to treat neurologic diseases. There is a controversy, whether intravenously administered human umbilical cord blood cells (HUCBC) migrate to the brain, differentiate and improve recovery after ischemia. In this study, $1-3 \times 10^6$ cells from non-cultured (non-committed) mononuclear HUCBC fraction were intravenously infused 1, 2, 3 or 7 days after a transient middle cerebral artery occlusion (MCAo) in adult rats. We found few human cells only in the ischemic area, localized mostly around blood vessels with few positive cells in the brain parenchyma. Timing of HUCBC delivery after ischemia or injection of Cyclosporin A at the time of delivery, had no effect on the number of human cells detected in the ischemic brain. Infusion of HUCBC did not reduce infarct volume and did not improve neurologic deficits after MCAo, suggesting that HUCBC failed to migrate/survive in the ischemic brain and did not provide significant neurological benefits.

Key words: umbilical cord blood cells, cell infusion, migration, MCAo, brain injury, neuroprotection

INTRODUCTION

Stem cells originated from bone marrow and cord blood can exhibit neuronal or glial cell markers under defined culture conditions *in vitro* (Sanchez-Ramos et al. 2000, Buzanska et al. 2002, Zigova et al. 2000, McGuckin et al. 2004) and have been shown to produce therapeutic effects in several animal models of neurological diseases, including stroke (Li et al. 2000, Chen et al. 2001a,b, Lu et al. 2002). Cord blood has emerged as an alternative source to bone marrow because of greater availability and weak immunogenicity. Several studies demonstrated behavioral benefit (Chen et al. 2001b, Vendrame et al. 2004), reduction of motor and neurological deficits (Chen et al. 2001b, Lu et al. 2002), and reduction of infarct volume (Borlongan et al. 2004, Vendrame et al. 2004) after intravenous infusion of

human umbilical cord blood cells (HUCB) in the rat middle cerebral artery occlusion model of stroke. Recent studies using neural-like stem cell line from human cord blood infused to ischemic animals, show a survival of transplanted cells in the cortex (Kozłowska et al. 2007), expression of neuronal marker and amelioration of neurological deficits (Xiao et al. 2005).

Despite the reported improvement of post-ischemic neurological outcome by administration of human cord blood cells in rodent models of stroke, a few of the administered cells could be demonstrated in brain parenchyma and even fewer expressed neuronal markers, raising a question as to the underlying mechanism. Using anti-human nuclei monoclonal antibody (MAB1281) Chen and other have found intravenously injected HUCBC in the ischemic brain at 14 and 35 days after transplantation (Chen et al. 2001a). They found a significantly higher number of HUCBC in the ipsilateral hemisphere, and some MAB1281-reactive HUCBC expressing neuronal, astrocytic and endothelial cell marker. Similarly, HUCBC cells were detected

Correspondence should be addressed to B. Kaminska,
Email: bozenakk@nencki.gov.pl

Received 08 September 2008, accepted 07 November 2008

by MAB1281 immunofluorescence and PCR analysis only in the injured brain hemisphere 4 weeks after MCAo and i.v. HUCBC infusion of HUCBC (Vendrame et al. 2004). The immunopositive cells were localized predominantly to blood vessels, with few cells being detected in the brain parenchyma. On the other hand, Borlongan and coauthors (2004) did not detect i.v. administered GFP-labeled HUCBC in the brains of animals at 1 to 3 days after stroke, even when cells were co-infused with the blood brain barrier permeabilizer (mannitol). Recent studies demonstrated that however, human stem cells enter the brain, they maintain a lineage commitment and do not adopt neural cell fates with measurable frequency (Massengale et al. 2005).

In the present study, we sought to evaluate whether HUCBC, delivered intravenously at various times after MCAo, enter the brain, survive and improve neurological functional recovery after a transient, focal ischemia in rats. An immunosuppressive drug Cyclosporin A (CsA) was applied together with HUCBC in some experiments to reduce a possibility of removing foreign cells by the immune system after systemic delivery. The infusion of HUCBC was performed at various times after MCAo to evaluate effects of hostile brain microenvironment on migration and maintenance of infused cells.

METHODS

Animals and surgical procedures

The experimental protocol was approved by the Local Animal Care and Use Committee and conforms to the national guidelines for the care and use of animals in research. Three-month old male, 270–320 g Wistar rats were used. Transient middle cerebral artery occlusion (MCAo) was induced with the intraluminal filament method as described before (Pera et al. 2004, 2005, Zawadzka and Kaminska 2005). Surgery was performed under halothane anesthesia (induction with 5% halothane, maintenance with 1.5%–2%, in oxygen). The right common, external and internal carotid arteries were exposed and 3–0 monofilament nylon suture was applied until mild resistance indicated occlusion of the MCA (ca. 18–20 mm). Rats were re-anesthetized 90 min later and the filament was withdrawn. Sham-operated animals underwent the same procedure, except of filament entry.

Neurological evaluation and infarct volume analysis

Neurological deficits resulting from the occlusion of MCA were verified by daily-performed somatosensory tests: the postural reflex and forelimb placing responses to visual, tactile, and proprioceptive stimuli (visual/tactile placing with frontal and lateral surface of paws) as described (Pera et al. 2004, 2005, Zawadzka and Kaminska 2005). Briefly, to examine the postural reflex rats were suspended by the tail and scored according to the following criteria: grade 0, no observable deficit; grade 1, forelimb flexion (hemiparesis); grade 2, decreased resistance to lateral push; grade 3, contralateral circling in addition to grade 2 deficits; and grade 4, no movement. Next, rats were held in a close distance from a table and the ability to extend their forelimbs toward the edge was measured to examine sensorimotor integration in forelimb placing responses (visual/tactile placing with frontal and lateral surface of paws, each tested separately): grade 0, no observable deficit; grade 1, partial or delayed placing (2 s); and grade 2, absence of paw placing. The maximal neurological score to obtain in the above tests was 12, indicating severe deficits.

Seven days after MCAo (or 14 days in longer term studies), rats receiving saline or i.v. HUCBC infusion, were deeply anesthetized with an overdose of sodium pentobarbital and the brains were fixed by transcardial perfusion with phosphate-buffered saline (PBS) and 4% paraformaldehyde (pH 7.4). Brains were immediately removed and postfixed. Serial free-floating sections of 25 μ m thickness were collected. For infarct volume analysis, selected sections were used for Nissl staining (0.5% thionine 37°C, 40 min). The infarct area in each brain slice was determined with the NIH program (ImageJ, v.1.29), total infarct volume was calculated and expressed as the percentage of total cerebral hemisphere volume (Swanson et al. 1990). Comparison between groups was made by one-way analysis of variance (ANOVA). Nonparametric data (neurologic deficit) were analyzed by the Kruskal-Wallis test (Statistica for Windows, version 6.0, StatSoft, Inc.). *P* value less than 0.05 was considered as statistically significant.

HUCBC isolation, administration and detection

Cord blood samples, collected from full-term normal deliveries, were diluted 1:1 with phosphate-buffered

saline (PBS) (Gibco). Subsequently, mononuclear cells were isolated by centrifugation on Ficoll (Sigma; 1.077 g/ml) at $400\times g$ for 40 min, washed twice and re-suspended in PBS with the addition of 0.5% human serum albumin (Biomed). Viability and cell quantification was determined by the trypan-blue dye exclusion method. A number of CD34+ cells in different samples was 2–2.5% as determined by flow cytometry as described (Buzanska et al. 2002).

Ischemic animals received an infusion of 3×10^6 mononuclear HUCBC or the same volume (1 000 μ l) of saline *via* the tail vein at 1, 2, 3, and 7 days after ischemia. In some experiments, ischemic animals receiving i.v. infusion 3×10^6 HUCB cells ($n=4$) were intraperitoneally injected with 10 mg/kg cyclosporin A (Novartis) at the time of HUCBC infusion.

Serial free-floating sections of 25 μ m thickness from perfused brains were deparaffinized in xylene, passed through a graded series of ethanol and washed for 5 min in water. Then, sections were treated with the blocking kit of Biotin/Avidin System reagents (Vector Laboratories, USA) and incubated overnight at 4°C with the MAB1281 (1:300, Chemicon International, Inc.) in PBS containing 2% BSA, 3% normal goat serum, 0.1% Triton X-100. Bound immunoglobulins were detected by incubation with biotinylated anti-mouse IgG (Vector, USA) followed by avidin D-fluorescein isothiocyanate (FITC) (Vector, USA).

After rinsing slices in PBS, sections were observed using fluorescent microscope (OLYMPUS IX-70). Immunohistochemical visualization was achieved with anti-mouse IgG conjugated to an alkaline phosphatase with a Fast Red chromogenic substrate system (Dako, USA).

RESULTS

The presence of human cells in rat brain sections was detected with anti-human nuclei antibody (MAB1281), which stains nuclei of all human cell types. Few human nuclei-specific MAB1281-positive cells were detected exclusively in the ipsilateral brain hemisphere of animals injected with 3×10^6 mononuclear HUCB cells at various times after MCA occlusion (Fig. 1). The immunopositive cells were localized predominantly around blood vessels, with few cells dispersed in the brain parenchyma. This low number of immunopositive cells was similar regardless whether HUCBC were infused at 1, 2, 3 and 7 days after MCAo (Table I). Two methods have been employed for detection of MAB1281-positive cells to ensure a correct and specific identification of HUCB cells. Immunosuppression with 10 mg/kg Cyclosporin A (CsA) did not increase the number of human immunoreactive cells present in the ischemic brain.

Table I

Summary of the results on detection of human immunopositive cells in the ischemic brain after infusion of HUCB cells at various times after MCAo

Type of cells	CsA	Time of infusion (days after MCAo)	Time of evaluation (days after MCAo)	Number of rats	Type of staining	Results
HUCBC	-	1	7	7	Anti-human nuclei(i) FastRed(ii) avidin-FITC	Few cells
HUCBC	-	2	7	3	Anti-human nuclei(i) FastRed(ii) avidin-FITC	Few cells
HUCBC	-	3	7	3	Anti-human nuclei(i) FastRed(ii) avidin-FITC	Few cells
HUCBC	-	7	14	3	Anti-human nuclei(i) FastRed(ii) avidin-FITC	Few cells
HUCBC	+	1	7	4	Anti-human nuclei(i) avidin-FITC	Few cells

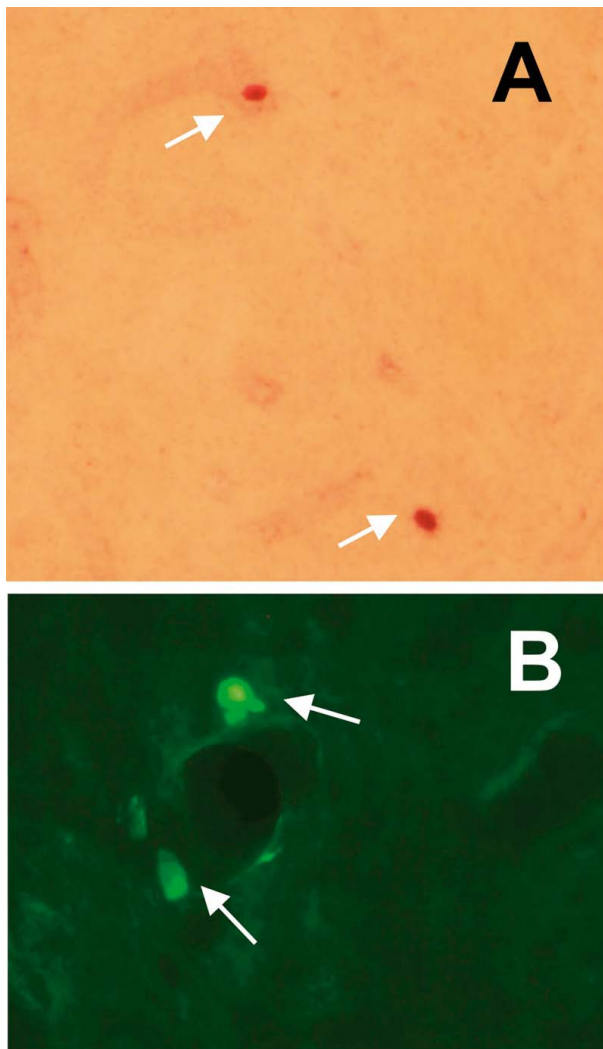


Fig. 1. Detection of HUCB cells in the ischemic brain 7 days after MCAo. 3×10^6 HUCBC were infused *via* a tail vein to ischemic rats 1 day after MCAo. Immunocytochemical detection of HUCBC with anti-Human Nuclei antibody was performed. Few positively stained HUCBC detected in the infarct area of the rat brain in the brain parenchyma (A) or around the vessels (B) 7 days after MCAo. Visualization was accomplished with anti-mouse IgG conjugated to an alkaline phosphatase with a Fast Red chromogenic substrate system (A) or biotinylated anti-mouse IgG followed by avidin D-fluorescein isothiocyanate (B).

Neurological scores in treated animals were evaluated at 1 and 7 days after MCAo ($n=15$). MCAO rats showed a partial spontaneous recovery in sensorimotor functions during the follow-up. Figure 2A shows total neurological scores in HUCBC *versus* saline-treated ischemic rats. No significant differences in neurological scores of HUCBC-treated MCAo ani-

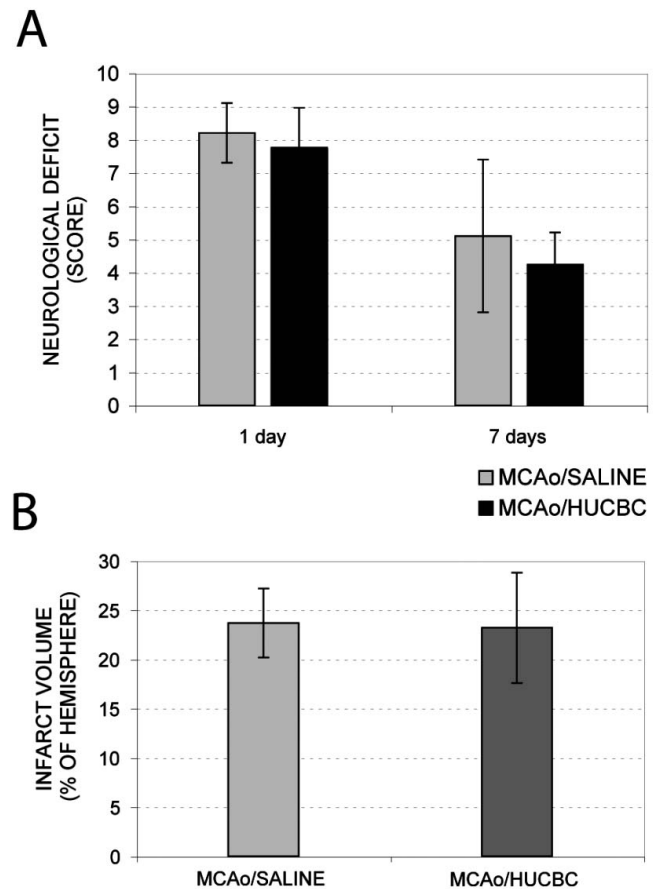


Fig. 2. Effects of systemic infusion of HUCB cells on neurological deficit scores and ischemic infarct volume after transient MCA occlusion in rats. The animals infused with 3×10^6 HUCBC or saline 1 day after ischemia/reperfusion, were killed 7 days after MCAo. (A) Neurological deficit scores were analyzed in somatosensory tests: the postural reflex and forelimb placing responses to visual, tactile, and proprioceptive stimuli (visual/tactile placing with frontal and lateral surface of paws), $n=15$. (B) The ischemic damage was visualized by a Nissl staining and the infarct area in each brain slice was determined with the ImageJ, v.1.29. Total infarct volume is expressed as the percentage of hemispheric volume and presented as mean \pm SEM; $n=5$. Comparison between groups was performed by one-way analysis of variance (ANOVA) using Statistica for Windows software.

mals were detected. Infarct volumes assessed from Nissl-stained sections on postoperative day 14 did not differ between the experimental groups. The striatum and cortex of the MCAO rats showed a vast tissue loss. An administration of HUCBC after MCAO did not reduce the extent of ischemic brain damage (Fig. 2B).

DISCUSSION

Stem cell transplantation is considered as a potential therapy which may improve outcome and recovery after stroke. Transplanted cells could replace cells that are damaged by ischemia and take over their function, either transplanted cells could secrete trophic factors that help to maintain surviving cells or otherwise enhance the local environment to improve functional recovery.

The migration of systemically delivered HUCBC to the injured brain and improvement of functional recovery are still controversial. Previous studies demonstrated that HUCBC infused i.v. after MCAo, survived and were distributed throughout the damaged brain of recipient rats, with the vast majority of cells localized to the ischemic area and few cells observed in the contralateral hemisphere (Chen et al. 2001b, Vendrame et al. 2004). In the present study, using antibody recognizing human nuclei and two different methods of detection, we have found only few immunopositive cells in the injured brain. The timing of delivery of HUCB cells after ischemia (1, 2, 3 or 7 days) had no influence on the number of detected cells. Immunopositive cells were detected exclusively in the ischemic area, predominantly around blood vessels. It suggests that HUCBC may be following homing signals that attract them to the injured site.

Several studies demonstrated that HUCBC administered after ischemia migrate, produce behavioral and anatomical recovery, and protect neural tissue from progressive damage (Chen et al. 2001b, Vendrame et al. 2004, 2005, Newman et al. 2005). Some studies suggest that the cells do not migrate to the site of injury (Borlongan et al. 2004) or do not exert their effects by engraftment, even though they migrate to the site of injury (Vendrame et al. 2005). Decreasing inflammation (Vendrame et al. 2005) or delivery of therapeutic molecules secreted by HUCBC (Borlongan et al. 2004) are considered as possible mechanisms underlying HUCBC neuroprotective effect.

In the present study, we have applied similar conditions as reported before (that is 3×10^6 intravenously administrated HUCB cells, 1 or 3 days after MCAo; detection of human cells using anti-Human Nuclear Antigen MAB1281 on perfused brain slices), but we could find in the brain only few HUCB cells per animal. Accordingly, we did not observe an improvement of neurological recovery or decrease in the infarct volume after intravenous infusion of HUCBC. Results of the present study demonstrate a lack of significant HUCBC migration to the injured brain area after a transient ischemia

and an evident lack of therapeutic benefits of intravenously injected HUCBC. Detection of only few HUCBC by human nuclear antigen immunoreactivity suggests their infrequent entry to the brain with localization limited mostly to the cerebrovasculature. It led to the conclusion that human umbilical cord blood cells are not able to populate the ischemic brain and protect neural tissue. Immunosuppression with CsA did not change the number of detected human immunopositive cells.

The presented results are similar to those described by Mäkinen and others (2006). They studied sensorimotor functions in rats subjected to 2 h MCAo using the limb-placing, beam-walking and cylinder tests at 4, 12, and 20 postoperative days and found that the recovery profile was similar in MCAO rats receiving PBS or HUCB cells. Infusion HUCBC did not affect impaired Morris water-maze performance of MCAO rats. They found only few human nuclei-specific MAB1281-positive cells in the ipsilateral hemisphere in MCAO rats that received HUCB cells and infarct volumes did not differ between the experimental groups. Furthermore, Nystedt and colleagues (2006) showed that an intravenous administration of human cord blood CD34+ cells 24 h after a transient or permanent MCAo did not change significantly recovery profile in rats. Infarct volumes assessed from Nissl-stained sections on postoperative day 25 did not differ between the experimental groups and MAB 1281-positive cells were not detected in the brain of MCAO rats that received CD34+ cells. A trend toward improved water-maze performance by CD34+ recipients that suggests some improvement of functional outcome in MCAO rats after systemic infusion of CD34+ cells.

Taken together, our results as well as studies by others (Mäkinen et al. 2006, Nystedt et al. 2006) demonstrate that HUCB cells do not improve functional recovery or histological outcome in MCAO rats after systemic administration because of limited migration of those cells into the ischemic brain.

CONCLUSIONS

Few human nuclei-specific (MAB1281)-positive cells were detected in the ipsilateral hemisphere in MCAO rats receiving human umbilical cord blood cells that suggests an infrequent entry of HUCB cells to the brain. Intravenously infused human umbilical cord blood cells do not populate and/or survive in the ischemic brain, and do not protect neural tissue against ischemia-induced damage in MCAo rats.

ACKNOWLEDGEMENT

The work was supported by PBZ-501/Z/13/7/2002 from the Ministry of Science and Higher Education (Poland)

REFERENCES

- Borlongan CV, Hadman M, Sanberg CD, Sanberg PR (2004) Central nervous system entry of peripherally injected umbilical cord blood cells is not required for neuroprotection in stroke. *Stroke* 35: 2385–2389.
- Buzańska L, Machaj EK, Zabłocka B, Pojda Z, Domańska-Janik K (2002) Human cord blood-derived cells attain neuronal and glial features in vitro. *J Cell Sci* 115: 2131–2138.
- Chen J, Li Y, Wang L, Zhang ZG, Lu D, Lu M, Chopp M (2001a) Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. *Stroke* 32: 1005–1011.
- Chen J, Sanberg PR, Li Y, Wang L, Lu M, Willing AE, Sanchez-Ramos J, Chopp M (2001b) Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. *Stroke* 32: 2682–2688.
- Kozłowska H, Jabłonka J, Janowski M, Jura M, Kossut M, Domańska-Janik K (2007) Transplantation of a novel human cord blood-derived neural-like stem cell line in a rat model of cortical infarct. *Stem Cells Dev* 16: 481–488.
- Li Y, Chopp M, Chen J, Wang L, Gautam SC, Xu Y, Zhang ZG (2000) Intrastriatal transplantation of bone marrow stromal cells (MSCs) improves functional recovery after stroke in adult mice. *J Cereb Blood Flow Metab* 20: 1311–1319.
- Lu D, Sanberg PR, Mahmood A, Li Y, Wang L, Sanchez-Ramos J, Chopp M (2002) Intravenous administration of human umbilical cord blood reduces neurological deficit in the rat after traumatic brain injury. *Cell Transplant* 11: 275–281.
- Mäkinen S, Kekarainen T, Nystedt J, Liimatainen T, Huhtala T, Närvänen A, Laine J, Jolkonen J (2006) Human umbilical cord blood cells do not improve sensorimotor or cognitive outcome following transient middle cerebral artery occlusion in rats. *Brain Res* 1123: 207–215.
- Massengale M, Wagers AJ, Vogel H, Weissman IL (2005) Hematopoietic cells maintain hematopoietic fates upon entering the brain. *J Exp Med* 201: 1579–1589.
- McGuckin CP, Forraz N, Allouard Q, Pettengell R (2004) Umbilical cord blood stem cells can expand hematopoietic and neuroglial progenitors in vitro. *Exp Cell Res* 295: 350–359.
- Newman MB, Willing AE, Manresa JJ, Davis-Sanberg C, Sanberg PR (2005) Stroke-induced migration of human umbilical cord blood cells: time course and cytokines. *Stem Cells Dev* 14: 576–586.
- Nystedt J, Mäkinen S, Laine J, Jolkonen J (2006) Human cord blood CD34+ cells and behavioral recovery following focal cerebral ischemia in rats. *Acta Neurobiol. Exp (Wars)* 66: 293–300.
- Pera J, Zawadzka M, Kaminska B, Szczudlik A (2004) Influence of chemical and ischemic preconditioning on cytokine expression after focal brain ischemia. *J Neurosci Res* 78: 132–140.
- Pera J, Zawadzka M, Kaminska B, Szczudlik A (2005) Neurotrophic Factor Expression after Focal Brain Ischemia Preceded by Different Preconditioning Strategies. *Cerebrovasc Dis* 19: 247–252.
- Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR. (2000) Adult bone marrow stromal cells differentiate into neural cells in vitro. *Exp Neurol* 164: 247–256.
- Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR (1990) A semiautomated method for measuring brain infarct volume. *J Cereb Blood Flow Metab* 10: 290–293.
- Vendrame M, Cassady J, Newcomb J, Butler T, Pennypacker KR, Zigova T, Sanberg CD, Sanberg PR, Willing AE (2004) Infusion of human umbilical cord blood cells in a rat model of stroke dose-dependently rescues behavioral deficits and reduces infarct volume. *Stroke* 35: 2390–2395.
- Vendrame M, Gemma C, de Mesquita D, Collier L, Bickford PC, Sanberg CD, Sanberg PR, Pennypacker KR, Willing AE (2005) Anti-inflammatory effects of human cord blood cells in a rat model of stroke. *Stem Cells Dev* 14: 595–604.
- Xiao J, Nan Z, Motooka Y, Low WC (2005) Transplantation of a novel cell line population of umbilical cord blood stem cells ameliorates neurological deficits associated with ischemic brain injury. *Stem cells Dev* 14: 722–733.
- Zawadzka M, Kaminska B (2005) A novel mechanism of FK506-mediated neuroprotection: downregulation of cytokine expression in glial cells. *Glia* 49: 36–51.
- Zigova T, Song S, Willing AE, Hudson JE, Newman MB, Saporta S, Sanchez-Ramos J, Sanberg PR (2002) Human umbilical cord blood cells express neural antigens after transplantation into the developing rat brain. *Cell Transplant* 11: 265–274.