Intrathecal injection of human umbilical cord blood stem cells attenuates spinal cord ischaemic compromise in rats†

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Abstract

OBJECTIVES: Spinal cord ischaemia with resulting paraplegia remains a devastating and unpredictable complication after thoraco-abdominal aortic surgery. With the advent of stem cell therapy and its potential to induce nervous tissue regeneration processes, the interest in the use of these cells as a treatment for neurological disorders has increased. Human stem cells, derived from the umbilical cord, are one of the strong candidates used in cell therapy for spinal cord injury because of weak immunogenicity and ready availability. We sought to evaluate the use of human umbilical cord blood stem cells (HUCBSCs) to attenuate the neurological effects of spinal cord ischaemia induced by high thoracic aorta occlusion.

METHODS: Forty Wistar rats were randomized to receive intrathecal injection of 10 µl phosphate buffered saline (PBS) solution containing 1 × 10⁴ HUCBSCs, 30 min before (Tpre group: n = 10) and 30 min after (Tpos group: n = 10) descending thoracic aorta occlusion by intraluminal balloon during 12 min. Control groups received only PBS solution (Cpre group: n = 10; and Cpos group: n = 10). During a 28-day observational period, motor function was assessed by a functional grading scale (Basso, Beattie and Bresnahan). Segments of thoracolumbar spinal cord specimens were analysed for histological and immunohistochemical assessment for detection and quantification of human haematopoietic cells (CD45⁺) and apoptosis (transferase-mediated deoxyuridine triphosphate-biotin nick-end labelling).

RESULTS: Overall mortality was 12 animals (30%). Therefore, the observational sample was composed of 28 animals. All groups showed similar incidence of paraplegia and mortality. The mean motor function scores showed no difference during time between the animals of each group, excepting for the Tpos group, which improved from 8.14 (±8.6) to 14.28 (±9.8) (P < 0.01). A treatment-by-time interaction was detected among animals that received HUCBSCs 30 min after ischaemia, with BBB scores higher from Days 14 to 28 compared with the first observational day with statistical difference (P = 0.01). Number of viable neurons was higher in the Tpos group (P = 0.14) and the incidence of apoptosis was lower in the same animals (P = 0.048), but showed no difference with its respective control. We confirmed the presence of CD45⁺ cells 4 weeks after intrathecal injection in both therapeutic groups but mainly in the Tpos group.

CONCLUSIONS: Intrathecal transplantation of HUCBSCs is feasible, and it improved spinal cord function, when they were delivered 30 min after spinal cord ischaemia, in a model of endovascular descending thoracic aorta occlusion in rats. Human umbilical cord blood is one of the potentially useful sources of stem cells for therapy of spinal cord ischaemia.

Keywords: Spinal cord ischaemia · Paraplegia · Stem cells · Thoracic aortic aneurysm

INTRODUCTION

Spinal cord ischaemia with resulting paraplegia remains a devastating and unpredictable complication after thoraco-abdominal aortic surgery [1]. Despite various surgical adjuncts and pharmacological interventions, the absence of a truly effective method for prevention and treatment poses a challenge to surgeons.

With the advent of stem cell therapy and its potential to induce nerve regeneration processes, including neuroregeneration, angiogenesis and synaptic plasticity [2], several models for treatment and prevention of neurological injuries have been proposed. Functional deficits caused by infrarenal aortic clamping were markedly ameliorated by intrathecal injection of bone marrow stem cells (BMSCs) in rabbits [3]. Using human spinal stem cells grafted directly into previously ischaemic spinal cord segments in rats, Cizkova et al. [4] showed a progressive recovery of motor function associated with neuronal differentiation and long-term survival of grafted neurons.

Since its clinical use in 1989, human umbilical cord blood stem cells (HUCBSCs) have been considered an interchangeable alternative to BMSCs. In addition to being readily available due to cord-blood banks, these cells are strong candidates for use in cell therapy for spinal cord disorders because of weak immunogenicity [5]. Intraspinal transplantation of HUCBSCs CD34⁺ improved
hindlimb functional recovery in adult rats after spinal cord hemisection [6] or contusion [7]. Saporta et al. [8] reported that intravenous infusion of unfractionated HUCBCs improved hindlimb function in a rat model of spinal cord compression. Recently, it was reported that intrathecal administration of these cells is feasible and capable of mitigating brain damage caused by ischaemia or neurodegenerative diseases [9].

No report appears to have investigated the use of unfractionated HUCBSCs intrathecally in a well-characterized spinal cord ischaemia rat model. To investigate the role of HUCBSCs in this model, we have examined the ability of these cells to engrant and survive in a zone of ischaemic lesion injury, as well as their efficacy to ameliorate the neurological deficit caused by a high-level occlusion of the descending thoracic aorta.

**MATERIALS AND METHODS**

The protocol was approved by our Ethics Committee for Analysis of Research Projects (CAPPesq) under the number 0281/09. All animals received humane care in compliance with the ‘Guide for Care and Use of Laboratory Animals’ (prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996).

**Experimental protocol**

Forty male Wistar rats, weighing 350–400 g, were randomized into four groups with 10 rats/group: the Tpre group received 10 µl of PBS 30 min after spinal cord ischaemia; the Tpos group received 10 µl of PBS solution containing 1 × 10⁶ HUCBSCs 30 min before spinal cord ischaemia; the Cpre group received 10 µl of PBS 30 min before spinal cord ischaemia; the Cpos group received 10 µl of PBS 30 min after spinal cord ischaemia.

**Acquisition and preparation of cells**

The human umbilical cord blood was collected from the umbilical vein of parturients with informed maternal consent in the Association Pro Matre of Rio de Janeiro. Procedure for separation of mononuclear cells and freezing was conducted in the laboratory of Cryopraxis, Inc. (Rio de Janeiro, RJ, Brazil). The samples containing a pool of 1 × 10⁶ stem cells in cryoprotective agent dimethyl sulfoxide (Sigma-Aldrich, São Paulo, SP, Brazil) were stored in tanks with liquid nitrogen and subsequently sent to the medical research laboratory, remaining frozen until its infusion.

Cells were thawed rapidly in a water bath at 37°C, diluted in a solution containing 3 ml of 20% human albumin (Sigma-Aldrich), 9 ml HemoHes 6% (B. Braun, São Paulo, SP, Brazil). The material was then centrifuged at 1200 rpm at 40°C for 10 min. The pellet was then resuspended in 1 ml of the solution and viability of the cells was accomplished by the trypan blue (0.4%) method. The final concentration was settled to 1 × 10⁶ in 10 µl.

**Animal model and surgical preparation**

All animals were anaesthetized in a chamber with 5% isoflurane, intubated and ventilated with a rodent ventilator (model 683, Harvard Apparatus, Holliston, MA, USA) at a 10 ml/kg tidal volume and 70 breaths/min frequency. They were maintained by inhalation of isoflurane during all the procedures. Rectal temperature was monitored with a probe (RET-2, Physitemp Instruments, Inc., Clifton, NJ, USA) and maintained at 37 ± 0.5°C with the Deltaphase Isothermal Pad (Braintree Scientific, Inc., Braintree, MA, USA). No intravenous drugs were administered.

Spinal cord ischaemia was induced by intraluminal balloon occlusion of the descending thoracic aorta jointly with left subclavian artery. Tail artery was cannulated with a polyethylene catheter (PE 10) to direct blood pressure control and heparine infusion. An arterial embolectomy catheter Fogarty 2F (Edward Lifesciences, Irvine, CA, USA) was advanced through an incision on the left common carotid artery to the descending thoracic aorta; through the same incision, a polyethylene catheter (PE 50) was introduced cranially to monitor the blood pressure that reflows through the internal carotid artery. This indirect proximal blood pressure control is necessary to achieve a controlled hypotension during aortic occlusion. After heparine infusion (100 U/kg), the catheter balloon was partly inflated and gently pulled to the left common carotid artery ostium, where it was completely inflated for 12 min. According to previous studies, this period is appropriate to bring about spinal cord ischaemia on the condition that there should be an added controlled systemic hypotension [10]. We applied concomitant controlled hypotension during aortic occlusion using a higher dose of isoflurane (5%) to achieve a distal media arterial pressure of ~10 mmHg.

**Intrathecal injection**

After anaesthesia, animals were placed on an operating surface that flexed the animal’s back. A small (1 cm) longitudinal incision was made over the L3–L5 spinous processes and the skin was retracted. A human neonatal lumbar puncture needle of 25 G (Becton Dickinson, Franklin Lakes, NJ, USA) was advanced into the spinal canal at the L3–L4 or L4–L5 level. Proper placement of the needle in the intrathecal space was indicated by three signs: loss of resistance at the time of entry (tentative sign), tail flick (more definitive sign) and presence of cerebral spinal fluid in the needle hub (most definitive sign). Once correct needle placement was confirmed, the solutions were injected over 1 min. The proper solutions for each group were injected 30 min before the spinal cord ischaemia induction in the Tpre and Cpre groups and 30 min after in the Tpos and Cpos groups.

**Neurological assessment**

During the 28-day observational period, motor function was assessed according to the scale of Basso, Beattie and Bresnahan (BBB) [11] by two independent observers, blinded regarding the experiment. According to this scale, animals can be classified into a score of 0–21 points—no movements to normal movement, balance and coordination, respectively. The first evaluation of motor function was performed 24 h after induction of ischaemia and every 48 h during the first week, and weekly afterwards.

**Histological and immunohistochemical assessment**

For the sacrifice and removal of the spinal cord, animals were deeply anaesthetized with pentobarbital 3% and decapitated with
consequent opening of the cervical spinal cord. The intact spinal cord was obtained by injecting cold saline solution in the distal cord canal; segments of the thoracolumbar transition (T2–L3) were sectioned and subsequently paraffin-embedded.

**Histological assessment**

Haematoxylin–eosin (H&E) staining was used to analyse neuronal cell death in grey matter. Three paraffin-embedded sections (4 µm) of thoracolumbar transition (T2–L3) were stained with H&E.

Ischaemic damage was characterized typically by necrotic neurons with eosinophilic cytoplasm and loss of cytoplasmic structures. Otherwise, when the cells demonstrated basophilic stippling (containing Nissl substance), the neuron cells were considered to be ‘viable or alive’.

The neurons in the half ventral grey matter (anterior and posterior horn) were counted by a blinded investigator, the media of the sections were obtained, and the changes detected were morphometrically treated, allowing comparison between the groups regarding the percentage of viable versus non-viable neurons.

**Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labelling staining method**

Transferase-mediated deoxyuridine triphosphate-biotin nick-end labelling (TUNEL) was used to identify apoptosis nervous cells. Briefly, after deparaffinization, sections were dipped in 10 nM Tris-HCl buffer, pH 7.4, containing 20 µg/ml proteinase K (Invitrogen, Grand Island, NY, USA) for 30 min at 37°C. Then the samples were coated with 10 ml of TUNEL solution (In Situ Cell Death Detection Kit Fluorescein, Roche Applied Science, Mannheim, BW, Germany) for 90 min at 40°C. The slides were washed in PBS (three times). The samples were mounted with the reagent Prolong Antifade Kit (Invitrogen, Grand Island, NY, USA) to preserve fluorescence. Cells with slightly stained nuclei or containing apoptotic bodies were considered to be apoptotic. Neurons whose nuclei exhibited very faint TUNEL staining and that did not contain apoptotic bodies were considered to be necrotic.

**Immunohistochemical detection and quantification of human haematopoietic cells CD45*RO**

The sections were rehydrated in a decreasing ethanol sequence for 2 min in each solution and blocked in solutions of 3% hydrogen peroxide in a darkroom. The slides were washed in distilled water and buffered in PBS solution for 5 min. To block non-specific protein, incubation with skimmed milk 10% Molico (Nestle, São Paulo, SP, Brazil) in distilled water for 30 min at room temperature.

After being blocked, the sections were incubated with the primary antibody, monoclonal mouse antihuman CD45*RO, clone OPD4, diluted 1:1000 (M0834, Dako, Carpinteria, CA, USA) in bovine serum albumin 1% overnight at 4°C, as a marker for transplanted human cells.

To proceed incubation with secondary antibody mouse antihuman antibody (NCL-END-Novocastra, New Castle Upon Tyne, UK), the slides were washed in PBS for 10 min and incubated for 15 min at room temperature, and then the reaction with the amplifier labelled with fluorescein was promoted for 15 min in the darkroom. The final step is incubation with antifluorescein antibody labelled with peroxidase CSAIL kit (Biotin-free tyramide signal amplification system, K1497, Dako, Carpinteria, CA, USA) for 15 min. The disclosure of the reaction is performed with 3,3-diaminobenzidine (0.4%) in PBS plus 1.2 ml of 3% hydrogen peroxide.

Slides were washed in water and counterstained with haematoxylin following the dehydration of the cuts in increasing ethanol sequence, cleared in xylene and mounted with Permout resin.

To determine graft survival semiquantitatively, a randomly selected section was prepared and counted by an investigator blinded to the experiment. For each section under analysis, the total numbers of positive cells for the grey matter were obtained. All images were made using a fluorescence microscope Axioshot 2 Plus (Zeiss, Göttingen, NI, Germany) with filter for fluorescein at ×40 magnification.

**Statistical analysis**

Data were analysed using the GraphpadPrism 6.0 statistical program. The parametric variables were expressed as mean value ± standard error of the mean (SEM) and analysed by one-way analysis of variance (ANOVA) or two-way ANOVA (group/time period), with repeated measures for functional analysis at different time periods. Bonferroni’s test was used as a post hoc test with adjustment for multiple comparisons. The non-parametric variables were expressed as median and percentile and analysed by the Kruskal–Wallis method, complemented by Dunn’s multiple comparisons test. The level of significance was set at 0.05.

**RESULTS**

**Physiological parameters**

Proximal and distal mean arterial pressure (dMAP) and indirect proximal mean arterial pressure (pMAP) at different times are given in Table 1. The interruption of blood flow through the descending aorta for 12 min resulted in a predictable change in arterial blood gas values, mainly characterized by decreased HCO₃⁻ (19.0 ± 3.5 to 12.6 ± 2.4) and increased lactate (3.1 ± 1.3 to 5.8 ± 1.7), with no significant variation among all the groups.

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<th>Table 1: Mean arterial blood pressure</th>
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<td>0 min 89.6 ± 11.9</td>
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<td>4 min 95.2 ± 2.0</td>
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<td>8 min 83.1 ± 1.3</td>
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Data are presented as mean ± standard mean error of 10 animals/group. MAP: mean arterial pressure; PAM: pulmonary arterial mean.
Neurological outcome

The overall mortality was 12 animals (30%), equally distributed among all groups (three animals per group), and the dead animals were discarded. The deaths were mostly attributable to seizures and visceral ischaemia, occurring within 72 h after operation. The observational sample was composed of 28 animals, following the criteria for postoperative care for animals with spinal cord injury [12]: 4 animals, Tpre (2), Cpre (1) and Tpos (1), were sacrificed before the end of the evaluation period, with 24 animals making it to the final observational period: Cpre (n = 5), Tpre (n = 5), Cpos (n = 8) and Tpos (n = 6).

Eighteen animals (64.2%) showed early severe or moderate neurological deficit (BBB of < 14), the remaining 10 (35.7%) presented a mild degree of motor dysfunction. The distribution was similar between groups. The average neurological scores of the groups during the follow-up are shown in Fig. 1. No significant functional recovery was found 28 days later, except for the Tpos group, which improved from 8.14 (± 8.6) to 14.28 (± 9.8) (P < 0.01). A treatment-by-time interaction was detected among animals that received HUCBSCs 30 min after ischaemia, with BBB scores higher from Days 14 to 28 compared with the first observational day, with statistical difference (P = 0.01). However, no marked functional recovery was found when the cells were delivered 30 min before ischaemia.

Histological assessment

Although a greater number of viable neurons were preserved in the Tpos group, compared with the control group, the difference did not reach statistical significance; the results of counting viable neurons are summarized in Fig. 2. Apparent neuronal damage was observed in all animals as evidenced by loss of viability by more than half of the neurons, mainly in T13 than in L2. The extent of ischaemic damage was grossly proportional to the neurological score.

Immunohistochemical assessment

Apoptosis findings by the TUNEL method are shown in Fig. 3. Individual values of apoptosis at the end of the observational period reveal low incidence of apoptosis, with no difference between the therapeutic groups and their respective controls. However, comparing the Tpos group with all other groups, there was a significant difference (P = 0.048) in favour of the Tpos group.

Survival of transplanted HUCBSCs–CD45+

A human membrane marker CD45⁺RO (clone OPD4) was identified 28 days after intrathecal injection of HUCBSCs in animals of the treatment groups, while controls showed negative marks. The number of the stem cells found in each group is shown in Fig. 4A and B.

DISCUSSION

Cell-based strategies are of particular interest in neurological conditions because mature spinal cord has limited capacity for self-repair. The results of this study demonstrated that HUCBSCs introduced intrathecally, in a spinal cord ischaemia rat model, engraft into an area of ischaemic lesion, survived at least for 4 weeks and were able to ameliorate some neurological deficit, when they were administered 30 min after a high-level occlusion of the descending thoracic aorta.

Cizkova et al. [4] showed a progressive recovery of motor function over 2–3 months after grafting human neural stem cells (HNSCs) in a spinal region-specific of a well-defined rat model of ischaemic spastic paraplegia. The improvement in motor function was associated with long-term survival of grafted neurons, neuronal differentiation and development of neuronal GABAergic phenotype in a subpopulation of grafted cells. There was also a correlation between the number of cell grafts and functional recovery.

Studies using BMSCs have demonstrated the feasibility of these cells being injected intrathecally, attenuating the neurological deficits during a 28-day recovering period, when they were delivered 2 days previously to spinal cord ischaemia [3]. The same outcome was reached when they were injected 2 or 24 h after an aortic occlusion,
but not 48 h later, stressing the importance of a therapeutic time window in cell therapy for spinal cord ischaemia [13]. Both the studies used an infrarenal aortic clamping model, which may not represent a truly effective model for thoracic aortic diseases [14].

Umbilical cord blood is an alternative and a rich source of human stem cells [15]. Because of its greater availability, owing to the increasing number of banks of human umbilical cord stem cells, weak immunogenicity and lower risk of mediating viral transmission, these cells are special candidates for use in spinal cord disorders. HUCBSCs have proven to be more advantageous than BMSCs in terms of cell procurement, storage and transplantation [16], moreover, the number and differentiation ability of BMSCs significantly decrease with age [17]. Neuronal lineages of stem cells (unipotent cells) probably have more capacity to become neurons and glial cells in vivo, but the requirement for using a growth factor and cell culture may eventually make their clinical use difficult.

The capabilities of HUCBSCs to engraft and survive in the injured area [8], differentiate into cells with neuronal phenotypes [18], express neural markers [19] and improve functional recovery in the spinal cord injury and stroke [20] were shown by different authors. Adult HUCBSCs also includes a spectrum of functional properties via mechanisms that may include replacement of damaged cells, neuroprotective effects, induction of axonal sprouting and neovascularization [21–23]. In our study, early improvement in function may result from neurotrophic effects that may modulate excitability and confer neuroprotection and anti-inflammatory responses. If neurogenesis and remyelination contribute to functional recovery, they would probably contribute to the later phase of recovery. The release of trophic factors by these cells may be sufficient to support damaged tissue, which may subsequently allow behavioural recovery [23]. Even without a specific test that confirms the differentiation of HUCBSCs into an astrocytic or neuronal phenotype, the morphology of the recovered stem cells that were seen 28 days after the spinal cord ischaemia did not show any specific features.

The rat model used in this study was reproducible, despite the concerns about mortality, mainly due to seizures and visceral ischaemia, as the blood flow not only to the spinal cord but also to liver, bowel and kidneys was dramatically reduced during the occlusion period. This is of importance because in other models of spinal cord ischaemia, where the aorta is cross-clamped below the renal arteries, these additional problems are occulted and can interfere with the level of neuronal damage in the spinal cord [14].

The route of cell administration is a key point in stem cell therapy. The need for development of effective cell-delivery methods to enhance the therapeutic efficacy of stem cells is pressing because the safety and efficacy of cell therapy depend on the mode of cell administration. Trials by intravenous injections of bone marrow-derived cells in rodents were reported, which is comparatively the least invasive approach and it is well tolerated [24]; however, many cells could be distributed widely throughout the body, such as in the liver, spleen and kidneys [25]. Further questions as to the reproducibility of this procedure have been raised.

We do not perform immunosuppression in light of the well-known harmful effects in the morbimortality of the animals and because the HUCBSCs have low immunogenicity and have been administrated intrathecally. Other authors have already realized the transplantation of these cells in a model of spinal cord injury without immunosuppression, with no clues of immunological rejection in the spinal cord [8].

Intrathecal injection seems to be a feasible and secure method to deliver these cells in spinal cord ischaemia. Additionally this is an easily reproducible method and allows for multiple injections, which could be useful for ascertain time-window therapy. Qualitatively, evidence of both necrosis and apoptosis was apparently higher in lumbar sections of control groups and in animals that received stem cells before ischaemia. The animals that were treated 30 min after ischaemia not only exhibited better neurological evolution but also demonstrated less histological damage, despite the absence of statistical significance.

Intrathecal injection of HUCBSCs is feasible and improved hindlimb motor function when they were delivered 30 min after spinal cord ischaemia in rats. Human umbilical cord blood is one of the potentially useful sources of cells for therapy of spinal cord ischaemia. In this study, the main limitations were related to number of animals and mortality. Another point is the observation period, which may not have been sufficient to evaluate a possible...
differ entiation of cells into neuronal lineages. Although further exploration is needed to ascertain issues such as the optimal therapeutic window time and the most appropriate cell type to be used for functional recovery, our study is one step towards using HUCBSC-based therapy to treat spinal cord ischaemia in humans as a complication of descending thoracic aorta cross-clamping.

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Conflict of interest: Gustavo Ieno Judas is research fellow of CAPES.

REFERENCES

APPENDIX. CONFERENCE DISCUSSION
Dr E. Quintana (Rochester, MN, USA): The authors report a spinal cord ischaemia model in rats and discuss the potential use of stem cells in this setting.

The results of your study suggest that the timing of injection may have an important influence on outcome. Although you are not able to provide a clear explanation of why stem cells seem to improve clinical condition, these results may open the door for future work. The fact that there was absence of differentiation into astrocytic or neuronal phenotype deserves special attention. We may need more follow-up to see if time allows for cellular differentiation.

I have just one question. Considering the experience with stem cells used in a myocardial setting where some patients experience arrhythmias after engrafting, do you expect any possible dangerous interaction of these cells in the spinal cord?

Dr Judas: We can have an interaction with the cells in the spinal cord in two ways: one, as you said, is about the differentiation of the cells in neuronal lineage cells. We don’t know if it happened here, because we didn’t analyse this hypothesis. We can make that ascertainment in the future. We may have had interaction here by neural mediators; we can hypothesize that happened here. So we expect that those cells interact with the spinal cord by mediator inflammatory response or something like that.

Dr K. Kallenbach (Heidelberg, Germany): I would like to ask you a question. If I understood correctly, you had a donor procedure where you obtained the umbilical cells. Those donors were Wistar rats as well? Where did you get the umbilical cord cells from?

Dr Judas: By donation of human umbilical cord blood.

Dr Kallenbach: It was human.

Dr Judas: Yes, just one patient. One cord blood is enough to get a high number of stem cells. And here we’re using a pool of stem cells not specifically matched.

Dr Kallenbach: Okay. Because this is a translational session. Where do you see your clinical application? Do you think if we have clinical signs of paraplegia, let’s say, after thoracoabdominal aortic replacement, do you imagine clinical application?

Dr Judas: We can monitor the evoked potential of the patient. If you have a sign of ischaemia of the spinal cord during the surgical procedure, you can place the cells because intrathecially it’s very simple way. You just have to have a catheter and you can infuse the cells. Or later in the ICU after the surgery if you have some sign of paraplegia, you can inject those cells. Because of this, this route of application is very interesting.

Dr Kallenbach: It sounds easy and we have to understand a little bit more how it works.