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Effect of age and gender on recovery after stroke in rats treated with bone marrow mononuclear cells

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ABSTRACT

Stroke is a disease of the elderly. However, most of the preclinical studies about the treatment of stroke with bone marrow-derived cells have used young animals. Here, it was assessed whether the sensorimotor recovery promoted by the treatment of the brain ischemia with the bone marrow mononuclear cells (BMMCs) is influenced by age and/or gender. Unilateral cortical ischemia by thermocoagulation was made in the primary motor and sensorimotor cortices in young and middle-aged rats of both genders. Twenty four hours after ischemia, animals received intravenous injection of BMMCs or vehicle. Each combination of age and gender received BMMCs from donor with the same combination. Survival rate and ischemic lesion size were quantified. Sensorimotor recovery was evaluated by the cylinder and adhesive tests. The results showed that the treatment with BMMCs resulted in sensorimotor recovery of both young and middle-aged ischemic rats. No important effect of gender was found, but age was a significant factor. Middle-aged animals had increased mortality and lesion sizes. In the adhesive test, middle-aged animals had lower BMMCs-induced sensorimotor recovery. The results suggest that the treatment of stroke with the BMMCs should be beneficial for males and females in the elderly.

1. Introduction

The treatment of stroke with bone marrow-derived cells has been shown to be effective in preclinical models of this disease (Mendez-Otero et al., 2007; Mezey, 2007). Moreover, several clinical trials are in progress (Savitz et al., 2011; Banerjee et al., 2012; Rosado-de-Castro et al., 2013), and it is expected that the efficiency of these cells in the treatment in humans may be known in the coming years. However, there are some questions about this treatment that were not answered by the preclinical studies, and the research with animal models is still needed. For example, most preclinical studies have tested the therapy with bone marrow-derived cells using young animals, but stroke is a disease that typically affects middle-aged and old individuals (Buga et al., 2013). Given the natural decline of the endogenous processes of regeneration and repair over the lifetime, considering the positive results in studies with young animals might not be appropriated to design therapeutic approaches for middle-aged and old humans (Li et al., 2010; Manwani et al., 2011; Buga et al., 2012). Furthermore, there are evidences that the gender is also a relevant factor on the level of injury, outcome and effect of therapies (Alkayed et al., 2000; Benice et al., 2006; Gokcay et al., 2011).

One of the main reasons for the difference in the brain tissue response to an ischemic insult among young and old individuals is the level of response of the brain innate immune system. The aged brain has faster astroglial scar formation, increased microglial reactivity, and greater cytokine release, which impairs the axonal growth and neuronal tissue recovery (Lucin and Wyss-Coray, 2009; Manwani et al., 2011; Buga et al., 2013). Even the normal aged brain seems to maintain a chronic low-level inflammation (Buga et al., 2013). Moreover, the hormonal difference among males and females influences the level of the ischemic brain injury (Alkayed et al., 2000), which might indicate hormonal influence in the innate immune system response and neuroprotective pathways.

Therefore, the inclusion of older animals and the comparison between genders in the experimental designs are needed to assess the actual potential of therapies for stroke. Surprisingly, few studies

Abbreviations: BMMCs, bone marrow mononuclear cells; MAF, middle-aged females; MAM, middle-aged males; PBS, phosphate-buffered saline; PID, post-ischemic days; SHR-SP, stroke-prone spontaneously hypertensive rats; TTC, 2,3,5-triphenyl tetrazolium chloride; YF, young females; YM, young males.

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about the treatment of brain ischemia with bone marrow-derived cells have included older animals (Shen et al., 2007; Brenneman et al., 2010; Taguchi et al., 2011; Wagner et al., 2012).

Some of the preclinical investigations with bone marrow-derived cells have demonstrated the therapeutic potential of the bone marrow mononuclear cells (BMMCs) (Iihoshi et al., 2004; Kamiya et al., 2008; Giraldi-Guimarães et al., 2009; Brenneman et al., 2010; Yang et al., 2011; Wagner et al., 2012; Sampaio et al., 2013). This bone marrow cell fraction contains monocytes, lymphocytes, mesenchymal and hematopoietic stem cells, and hematopoietic and endothelial progenitor cells (Orkin, 2000; Weissman et al., 2001; Wang et al., 2008). BMMCs can be harvested in hours and administrated in the acute phase of stroke without cultivation (Iihoshi et al., 2004; Brenneman et al., 2010; Battistella et al., 2011).

Here, to evaluate whether the sensorimotor recovery promoted by the treatment with BMMCs can be influenced by age and/or gender, ischemic young and middle-aged rats of both genders were treated with BMMCs or vehicle and their sensorimotor performances were measured.

2. Materials and methods

2.1. Animals

Male and female Wistar rats aged, at the beginning of the experiment, between two and five months (young) or twelve and seventeen months (middle-aged; retired breeders) were used. All animals were housed in a colony room with controlled temperature, and with food and water available ad libitum. The experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Ethics Committee of our institution.

2.2. Surgery

Young males (YM), middle-aged males (MAM), young females (YF) and middle-aged females (MAF) were submitted to focal cortical ischemia by the method of the thermocoagulation of the blood in the submeningeal blood vessels of the primary motor and sensorimotor cortices, as previously described (Szele et al., 1995; Sampaio et al., 2013). Briefly, the animals were placed in a stereotaxic apparatus (Insight Ltda., Ribeirão Preto, SP, Brazil) after anesthesia with ketamine hydrochloride (100 mg/kg, i.p.) and xylazine hydrochloride (10 mg/kg, i.p.). The skull was surgically exposed, and a craniotomy was made, exposing the frontoparietal cortex contralateral to the preferred forelimb in the adhesive test (+2 to −6 mm A.P., and +or −2 mm to from bregma M.L.; Paxinos and Watson, 2005, See item 2.5). Blood was thermocoagulated transdurally by approximation of a hot probe to the dura mater, without touching it. The skin was then sutured, and the animals were returned to the colony room after recovery from anesthesia.

2.3. Quantification of the ischemic lesion extension

YM, MAM, YF and MAF were euthanized approximately 24 h after ischemia to verify the cortical lesion extension ($n = 4$ for each group). Brains were rapidly removed from the skull and sectioned in the coronal plane at 2 mm of thickness using a rat brain blocker/slicer (Insight Ltda.). The slices were immersed for 30 min into a 2% 2,3,5-triphenyl tetrazolium chloride (TTC) solution at 37 °C, and then washed in phosphate-buffered saline (PBS) and fixed in 10% formaldehyde overnight. Digital images were captured from reacted slices with a camera coupled to a dissecting microscope and to a PC computer. Lesion areas of slices were measured from digital images using specific tools of ImageJ software (NIH). The lesion area of each slice was multiplied by its thickness (2 mm), obtaining the volume (mm^3). For each animal, the total lesion volume was the sum of the lesion volumes of its slices. To verify possible differences in the brain size between the groups, the area of the slice located in the portion of greater brain thickness (around −5.88 mm from bregma; Paxinos and Watson, 2005) was also measured for each animal.

2.4. BMMCs transplantation

Naïve donor animals from each combination of age and gender (YM, MAM, YF and MAF) were used to obtain bone marrow, which was harvested aseptically from tibias and femurs, as previously described (Sampaio et al., 2013). Briefly, bone marrow was extracted from the bones and collected in sterile tubes with serum-free DMEM-F12 (Gibco Brl, Grand Island, NY, USA). Cells were mechanically dissociated, centrifuged and resuspended in serum-free DMEM-F12. Separation of mononuclear fraction was made by centrifugation in Histopaque 1083 (Sigma–Aldrich, St. Louis, MO, USA). Mononuclear cells were collected, washed with PBS, counted and resuspended in PBS.

The administration of BMMCs or vehicle (PBS) occurred approximately 24 h after ischemia. Ischemic animals were anesthetized with ketamine hydrochloride (100 mg/kg, i.p.) and xylazine hydrochloride (10 mg/kg, i.p.), and BMMCs or PBS were injected through the jugular vein. In all animals treated with BMMCs, the dose was approximately 3×10^7 cells. The ischemic animals and the BMMCs from the naïve donors, or only vehicle, were combined to form the following experimental groups for the behavioral analyses: YM/BMMCs group, ischemic YM that received BMMCs from YM donors; YM/control group, ischemic YM that received vehicle; MAM/BMMCs group, ischemic MAM that received BMMCs from MAM donors; MAM/control group, ischemic MAM that received vehicle; YF/BMMCs group, ischemic YF that received BMMCs from YF donors; YF/control group, ischemic YF that received vehicle; MAF/BMMCs group, ischemic MAF that received BMMCs from MAF donors; MAF/control group, ischemic MAF that received vehicle. The number of animals for each group and their ages are shown in Table 1.

2.5. Behavioral tests

We have already shown the high effectiveness of the cylinder test and the adhesive test (Schallert, 2006; Schaar et al., 2010) to assess sensorimotor function after thermocoagulatory cortical lesion (Sampaio et al., 2013). Thus, they were used in the present study to evaluate the functional recovery of the forelimb contralateral to the ischemic cortical hemisphere. All animals of each experimental group were submitted to both tests. The animals were tested one day before ischemia and at post-ischemic day (PID) 2, and then weekly. Pre-ischemic day was plotted in graphs as PID 0. As previously described (Sampaio et al., 2013), briefly:

(1) Cylinder test: the trial consisted in placing the animal inside a glass cylinder and count the supports in the wall with ipsilateral (to the lesion) forelimb, contralateral forelimb or simultaneous support with both forelimbs. For each animal at each PAD, the percentage relative to the total number of usages (ipsilateral + contralateral + simultaneous) was calculated for ipsilateral (unimpaired) and contralateral (impaired) uses. An asymmetry score for each animal was calculated at each PAD by the following formula: asymmetry score = (% of ipsilateral uses) − (% of contralateral uses).
(2) Adhesive test: a round adhesive paper (13 mm diameter) was placed on the inner portion of each wrist of the animal. One trial consisted in placing the adhesive papers and their