Effects of bone marrow mononuclear cells on induction of axonal sprouting in cortico-cortical and cortico-striatal pathways in an animal model of cortical ablation.

Maria de Fatima dos Santos Sampaio (✉ mfss.sampaio@gmail.com)

Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

Arthur Giraldi Guimarães

Universidade Estadual do Norte Fluminense Darcy Ribeiro

Camila da Silva Lourenço

Universidade Estadual do Norte Fluminense Darcy Ribeiro

Messias Gonzaga Pereira

Universidade Estadual do Norte Fluminense Darcy Ribeiro

Norberto Cysne Coimbra

Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

Research note

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Abstract

Many therapies have been proposed in order to investigate the mechanisms of neural repair associated with neurological diseases, including BMMC (bone marrow mononuclear cells) transplantation. However, studies show that some brain injuries are less responsive to neural repair, such as, for example, cortical ablation. On the other hand, models of cortical ablation have shown functional recovery after BMMC transplantation. Thus, it is relevant to expand knowledge about BMMC, also, in the induction of neuroplasticity, in this model, considering treating a promising event for the rehabilitation of neurological diseases. Using the experimental model of cortical ablation of the sensorimotor cortex, which is known to be poorly responsive to neuroplasticity, the aim of this study was to investigate the effects of BMMC on axonal sprouting induction in cortico-cortical and cortico-striatal projections. An anterograde neurotracer was used to evaluate the distribution of axonal fibers. Quantitative analyzes were performed and the results showed that BMMC were not able to significantly induce axonal sprouting in the evaluated areas. Our results reinforced the idea that cortical ablation may be less responsive to neuroplasticity and the beneficial effects of BMMC therapy depend on the particularities of a neural microenvironment intrinsic to a given lesion.

Introduction

Injuries to the motor cortex or corticospinal tract interfere with motor control, making movement generation and coordination difficult and causing functional losses. [1]. Many therapies have been proposed to investigate the mechanisms of neural repair, including bone marrow mononuclear cells transplantation (BMMC).

BMMC consist of hematopoietic progenitor cells and hematopoietic stem cells, lymphocytes, monocytes and a small number of mesenchymal stem cells (MSCs). They have shown beneficial effects by presenting the ability, among others, to release trophic factors and cytokines that favor neuroprotection and repair of injured tissue and sensorimotor functional recovery [2–7].

One of the important events seen as associated with rehabilitation after CNS injury is neuroplasticity. However, it is known that this process is different for each injury [8–13].

In a previous study [14], our group investigated the effects of BMMC on functional recovery after focal cortical ablation in rats. Given the scientific evidence about neuroplasticity [15–17], we considered the instigating cortical ablation model to evaluate the efficiency of BMMC in the induction of brain plasticity. Thus, this study aimed to investigate the effects of BMMC on induction of axonal sprouting in cortico-cortical and cortico-striatal projections. For this, an anterograde neurotracer was used to evaluate the distribution of axonal fibers in areas of the neocortex and ipsi neostriatum and contralateral to the lesion.

Methods

Animals and Surgery

A total of 30 adult male Wistar rats (weighing 320–400 g), provided by the Darcy Ribeiro Northern Fluminense State University (UENF), with 3–4 months of age. All experimental protocols were also approved by the Commission of Ethics in Animal Experimentation of UENF (process 086/ 2010) on 09/02/2010. Rats were housed in groups of four per cage with food and water available *ad libitum* in a temperature-controlled room ($22 \pm 1^\circ\text{C}$) under a 12 h/12 h light/dark cycle (lights on at 7:00 a.m.). Rats were transported to the experimental room in their home cages and left undisturbed for 1 h prior to the experiments.

Ablation was performed by cerebral cortex aspiration, as previously described [8]. Briefly, after anaesthesia with ketamine hydrochloride at 90 mg/kg, *i.p.* (Ketamine Agener, União Química Farmacêutica Nacional, Embu-Guaçu, SP, Brazil; 0.2 mL of 10% solution) and xylazine hydrochloride at 10 mg/kg, *i.p.* (Dopaser®, Hertape/Calier, Juatuba, Minas Gerais, Brazil), the left frontoparietal cortex (+ 2 to – 6 mm A.P. from the bregma) was exposed and aspirated with a pipette tip (1 ml) attached to a vacuum pump. A piece of collagen hemostatic sponge was put inside the lesion, the skin was sutured, and the animals were kept warm under a hot lamp and returned to colony room after recovery from anaesthesia.

BMMC obtaining and transplantation

Bone marrows were obtained from donor naïve rats ($n = 3$), as previously described [2]. The animals were euthanized with a lethal dose of ketamine hydrochloride (*i.p.*) and xylazine hydrochloride (*i.p.*). BMMCs were collected and washed with phosphate-buffered saline (PBS). Following cell count, they were re-suspended in sterile physiological saline, and the final concentration was approximately 3×10^7 BMMCs/0.5 mL.

Twenty four hours after surgical procedure, animals were anaesthetised with ketamine hydrochloride (90 mg/kg, *i.p.*) and xylazine hydrochloride (10 mg/kg, *i.p.*) and injected through the left jugular vein with BMMC or vehicle (PBS). The skin was sutured, and the animals were kept warm under a hot lamp and returned to colony room after recovery from anaesthesia.

Experimental design

The animals were randomly divided into experimental groups. NAÏVE (animals without lesion), CONT (animals submitted to cortical ablation and treated with vehicle), BMMC (animals submitted to cortical ablation and treated with mononuclear cells of bone marrow). The therapeutic window was chosen based on our previous studies [5].

Animals from the NAÏVE ($n = 7$), CONT ($n = 6$) and BMMC ($n = 9$) groups were subjected to anterograde tracer microinjections in sixty days after the surgical procedure. In all groups, the animals were euthanized 8 days after the tracer microinjections.

Characterisation of the lesion

For the characterisation of the lesion, four untreated animals were euthanised 72 h after cortical ablation with a lethal dose of ketamine hydrochloride (i.p.) and xylazine hydrochloride (i.p.). The brain was rapidly, according Sampaio et al [5]. The slices were immersed for 30 min into 2% 2,3,5-triphenyltetrazolium chloride (TTC, T8877/1 Sigma-Aldrich) solution at 37 °C. Digital images were captured from reacted slices with a camera coupled to a dissecting microscope and to a computer.

In vivo neural tract tracing procedure

Sixty days after cortical ablation, the animals were anaesthetised with xylazine hydrochloride (10 mg / kg, i.p.) and ketamine hydrochloride (90 mg / kg, i.p.), and fixed in a stereotaxic apparatus. A small trepanation was performed in the calvaria over the sensorimotor cortex in the hemisphere contralateral to the lesion. Using a stereotaxic coordinates obtained from a rat brain atlas (anteroposterior axis, + 1.2 mm, mid-lateral axis, -2.7 mm), the 10% AlexaFluor 546-conjugated biodextran amine (BDA) solution (Dextran, Alexa Fluor 546; 10,000 MW, Anionic, Fixable, Life Technologies, Austin TX, USA) was microinjected into the primary motor cortex (M1) through a Hamilton syringe. The injections were made in the depths of 1.8 mm and 2.0 mm from Bregma, with a total volume of solution injected of 0.5 µL.

Histological Analysis

After 8 days of the neural tract tracer injection, each animal was euthanized with a lethal dose of with ketamine hydrochloride (i.p.) and xylazine hydrochloride (i.p.). Immediately after this procedure, the encephalon was perfused through the left ventricle with physiological saline solution (NaCl, 0.9%), followed by 4% paraformaldehyde solution in phosphate buffer (0.2M, pH 7.4). The encephalon was rapidly removed and re-immersed in the fixative for 4 h and then incubated in 20% sucrose dissolved in phosphate buffer (0.2M, pH 7.4) for 24 hours at 10 °C. The encephalon was cut, soaked in a compound of 10.24 w/w polyvinyl alcohol and 4.26 w/w polyethylene glycol (Tissue Tek O.C.T.) and immersed in liquid nitrogen for freezing. It was then sectioned in the coronal plane (30 µm thick), at -20 °C in a cryostat (CM 1950 Leica, CM 1950 Leica, Wetzlar, Germany) and the slices arranged in gelatinised slides, which were subsequently visualised under a motorised fluorescence microscope (AxioImager Z1 with APOTOME II; Zeiss, Oberkochen, Germany). Statistical analysis was performed exclusively with data from the animals that presented evidence that microinjection in the primary motor cortex (M1) was successfully performed.

Data analyses

The images were selected and treated using the ImageJ software (NIH). From the efficacy of the neural tract tracing, in each two brain sections slides per animal, 3 fields were analysed as follow: Neocortex surrounding the lesioned area and the neostriatum ipsilateral and contralateral to the cortical brain lesion (Fig. 2J). The mean per animal was calculated in respective areas and then the mean for each experimental group was plotted for the statistical analyses. The percentage of axonal fibres was computed based on the number of axons identified per unit of visual field area.

Data from the experiment were submitted to the one-way ANOVA, followed by Tukey's post hoc test. Values were reported as the mean and standard error of the mean (S.E.M.). P-values < 0.05 were considered statistically significant. The distribution of the variables passed in the normality test (Shapiro-Wilk normality test).

Results

Histochemical characterisation of the cortical lesion

Using TTC, it was demonstrated that all lesioned animals, 72 h after each the surgical procedure, showed in the sensory-motor region, loss of neocortical tissue restricted to the sensory-motor cortical area when submitted to the cortical ablation, as shown in Fig. 1.

Quantitative analysis of cortico-cortical and cortico-striatal projections of naïve rats and those submitted to a unilateral cortical ablation after BMMC or vehicle transplantation

Results of quantitative morphological analyzes showed that intravenous treatment with BMMC did not significantly increase the number of BDA-positive axonal fibers in areas of the neocortex and ipsilateral neostriatum and contralateral to the lesion (Fig. 2A - I). Analysis of the neural tissue ipsilateral to the cortical lesion and surrounding the brain damage, according to the one-way ANOVA, there was a significant effect of the treatment ($F_{2,16} = 3.992$, $p < 0.05$). The neocortical lesion with cortical tissue ablation of animals not treated with BMMC (CONT group) was followed by a significant decrease in the number of BDA-labeled axonal fibers in the cortical layers surrounding brain injury compared to healthy rodents (NAÏVE group) (Tukey post hoc test; $p < 0.05$). These findings are shown in Fig. 2K.

Regarding the the neostriatum ipsilateral to the brain lesion, there was a significant effect of the treatment ($F_{2,13} = 6.444$; $p < 0.05$), according to one-way ANOVA. Control injured animals (CONT) showed a reduction in BDA-positive axonal fibers when compared to the healthy group (NAÏVE group). These data are shown in Fig. 2L.

With respect to the caudatum-putamen contralateral to the lesion, according to one-way ANOVA, there was any significant difference between the experimental groups ($F_{2,17} = 1.143$; $p > 0.05$), as shown in Fig. 2M.

Discussion

Our results showed that BMMC treatment did not induce an increase in axonal fibers in areas of the contralateral cortex and ipsilateral and contralateral neostriatal lesion, of animals injured by cortical ablation. There are few studies in the scientific literature regarding cell therapy in cortical ablation models, possibly because it represents a less frequent clinical situation when compared to stroke or head trauma. Cortical ablation resembles a condition of surgical removal of a brain tumor [14].

In approaches to neuroplasticity after injury, there is scientific evidence that cortical ablation has been shown to be poorly responsive to structural plasticity [9–11]. Therefore, it became an attractive model for this work, which aimed to extend the studies of BMMC efficiency, now contemplating the induction of neuronal circuit remodeling, in an attempt to better elucidate the relationship of neuroplasticity and motor functional recovery.

The results of this study corroborate the findings in the literature [11, 18] showing that after cortical ablation in a sensorimotor area there was little responsiveness to cortico-cortical and cortico-striatal neuroplasticity. In this study, we also observed that treatment with BMMC was not able to reverse this picture.

Carmichael and Chesselet [19] contributed to these findings, that different sensorimotor cortex lesions may promote different responses in the induction of axonal sprouting, even being injuries of the same location and extension. These authors, using ablation and ischemia models, showed that there was an increase in axonal marking in the medial cortex and striatum, both ipsilaterally to the lesion, but only in animals submitted to ischemia. Thus, attention was paid to a brain reorganization dependent on the type of injury induced in rats, reinforcing the previous results of Szele et al [8] who indicated that aspiration lesions promoted a greater inhibition of structural plasticity, unlike cortical ischemic lesions.

According to Freitas et al. [13], one of the possible explanations of not observing a significant response to structural plasticity after cortical ablation is due to the injury mechanism itself. Has been established that inflammation triggers injury-induced structural plasticity, and therefore pathologies such as stroke and TBI would be more responsive to this event as they cause substantial necrosis and favor a significant inflammatory response [11, 18, 20, 21].

Research has shown that BMMC have a neural neuroprotection and repair capability. Another important point to be considered in future research is that inflammatory processes, intrinsic to each lesion, may mediate neural repair and may inhibit or facilitate axonal sprouting following injury to the central nervous system. And, consequently, seek therapeutic strategies that positively interact with all these factors, inherent in each pathological process.

Limitation

This work was limited to analyzing only the axonal sprouting in cortico-cortical and cortico-striatal projections, since the main purpose of this study was to establish or not, a relationship between the functional recovery and neuroplasticity, already demonstrated in previous studies of ours research team. Therefore, it became evident that BMMC, despite being considered a promising therapy, was not able to induce neural repair in a model of cortical ablation.

Abbreviations

ANOVA

Analysis of variance
BDA
Biodextran amine
BMMC
Bone marrow mononuclear cells
CEUA
Commission of Ethics in Animal Experimentation
CNS
Central nervous system
M1
Primary motor cortex
MSCs
mesenchymal stem cells
PBS
Phosphate buffered saline
TTC
2,3,5-triphenyltetrazolium chloride

Declarations

Availability of data and materials

Data supporting our finding are available from the corresponding author.

Ethics approval and consent to participate

All experimental protocols were also approved by the Commission of Ethics in Animal Experimentation (CEUA) of Darcy Ribeiro Northern Fluminense State University, process 086/2010 on 09/02/2010 and process 151/2012 on 24/04/2012.

Consent for publication

"Not applicable" in this section.

Competing interests

The authors declare that they have no competing interests"

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Authors' contributions

Maria de Fátima dos Santos Sampaio performed the experiments, analysed and interpreted the data, performed the statistical analysis, designed the figures and wrote the manuscript. Arthur Giral di Guimarães designed the experiments. Camila da Silva Lourenço performed the experiments. Messias Gonzaga Pereira performed the statistical analysis and reagents. Norberto Cysne Coimbra designed the experiments, analysed and interpreted the data, wrote the manuscript. All authors have approved the final version of the manuscript.

Corresponding author:

Dr. Maria de Fátima dos Santos Sampaio (mfss.sampaio@gmail.com). Laboratory of Neuroanatomy and Neuropsychobiology, Department of Pharmacology, Ribeirão Preto Medical School of the University of São Paulo (FMRP-USP), Av Bandeirantes, 3900, Ribeirão Preto, 14049-900, São Paulo, Brazil.

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Figures

Figure 1

Extension of the cortical ablation procedures. The figure shows sequential coronal sections (2 mm) of Wistar rat brains 72 hours after each cortical lesion, submitted to the histochemical reaction with 2,3,5-triphenyltetrazolium chloride (TTC), which reddens the viable tissue. (A - D) Histological sections of a brain reacted after ablation, observing the removal of the sensorimotor area (white arrowheads in the top).

Figure 2

Photomicrographs of transverse sections of "NAÏVE" Wistar rats brain (A-C), of "CONT " Wistar rats brain (D-F), of "BMMC " Wistar rats brain (G-I). Representative site of injection of the anterograde AlexaFluor 546-conjugated biodextran amine (BDA) neurotracer in the primary motor cortex (A), fibres of passage in the external pyramidal layer (B), corpus callosum to connect the contralateral (D and G), cerebral cortex ipsilateral to lesion (E), internal pyramidal layer (H) and fusiform layer. (C,F and I). Representative site of injection scheme of the anterograde AlexaFluor 546-conjugated biodextran amine (BDA) neurotracer in the primary motor cortex, demonstrating the quantified areas for analysis of cortico-cortical and cortico-striatal projections (J). The effect of the transplantation of BMMC or vehicle-treatment on axonal sprouting in cortical layers surrounding the brain lesions (K) and in the neostriatum ipsilateral (L) and contralateral (M) to the cortical ablacion were also demonstrated. Data were represented as mean ± S.E.M. (n= 6-9 rats per group). ANOVA (one-way) followed by Tukey's post hoc test. *p <0.05, in comparison to the NAÏVE group. Arrowhead indicate BDA-labelled neuronal fibres, transparent arrow indicate site of injection of the anterograde AlexaFluor 546-conjugated biodextan amine (BDA)

neurotracer in the primary motor cortex (M1) and white arrows indicate BDA-labelled perikarya located in different brain structures. Scale bars: 200µm on the panel A; 20µm on the panels B, C, D, E, F, G, H and I.

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