Convulsive Seizure Protection after Hippocampal Transplantation of Mesenchymal Cells from Adipose Tissue in Mice

Tamura BP[1#], Almeida DC[2#], Felizardo RJ[2], Olanda GC[1], Bocca LF[1], Pinhal NS[1], Alves-de-Moraes LBC[1], Covolan L[1], Câmara NOS[2,3] and Longo BM[1*]

#Authors contributed equally to this work

[1]Laboratory of Neurophysiology, Department of Neurophysiology, Federal University of São Paulo - UNIFESP – São Paulo, Brazil.
[2]Laboratory of Clinical and Experimental Immunology, Department of Medicine – Nephrology, UNIFESP – São Paulo, Brazil.
[3]Laboratory of Transplant Immunology, Department of Immunology, University of São Paulo – USP, Brazil

Abstract

A considerable number of epileptic patients had become resistant to antiepileptic drugs, justifying the need for development of new therapeutic strategies to treat epilepsy. The use of mesenchymal stem cells is an innovative and accessible strategy for the treatment of neuronal disorders, due to their involvement in immunoregulatory mechanisms, trophic and anti-apoptotic action.

Objective: Based on this evidence, we evaluated the protective effect of mesenchymal cells from adipose tissue (MCAT) by behavioral and inflammatory responses against convulsive seizure induced by maximum electroconvulsive shock (MES).

Methods: MCAT cells were transplanted into the hippocampus of adult male mice, and ten days after the transplantation MES stimulation was applied to induce a generalized tonic-clonic seizure. To evaluate the anticonvulsant activity of MCAT cells, we evaluated the parameters involved with: protection and reduction in the duration of tonic phase, reduction in the mortality rate, and alteration in the hippocampal gene expression of IL-1beta, IL-6, IL-4, IL-10, caspase-1, iNOS and TNFα.

Results: MCAT cells transplanted into the hippocampus altered the convulsive threshold, showed anticonvulsant effect by protecting from tonic seizures and mortality and reduced the hippocampal expression of transcripts related to inflammatory response such as IL-1beta, IL-6, caspase-1 and iNOS and increased the level of anti-inflammatory interleukin IL-4.

Conclusion: The anticonvulsant effects of the MCAT cells on acute convulsive seizure may be related to inhibitory factors and immunomodulatory mechanisms assigned to mesenchymal cells in the hippocampus. These anticonvulsants mechanisms of MCAT cells bring strong therapeutic implications for the control of epileptic seizures.

Keywords: Epilepsy; Maximum electroconvulsive shock; Hippocampus; Cell therapy; Mesenchymal cells from adipose tissue; Immunomodulatory mechanisms; Interleukins

Introduction

Treatments to control epileptic seizures are practically restricted to the use of antiepileptic drugs such as phenytoin, carbamazepine and oxcarbamazepine that present many secondary effects and are not totally effective in controlling the seizures. A considerable number of patients diagnosed with epilepsy had become resistant to these drug treatments. Moreover, epileptic seizure has been proposed as an inflammatory event, and that inflammatory mediators may contribute to the onset and recurrence of seizures [1]. In this context, it is essential to develop new therapeutic approaches for controlling or even suppressing the convulsive seizures through new research fronts, including the use of mesenchymal stem cell-based therapy.

An increasing number of studies have shown the therapeutic potential of stem cells obtained from various sources such as bone marrow, brain, umbilical cord blood, skin and adipose tissue, most of them proposed to be used in treatments of central nervous system diseases [2]. Mesenchymal stem cells (MSCs) present in the stroma of various vascularized organs represent one of the most studied populations of adult stem cells. Advantageously, these cells can be obtained from different tissues and expanded in vitro, as well as their trophic properties, make these cells excellent candidates for use in cell-based therapy [3]. Adipose tissue is an accessible source of progenitor mesenchymal cells that has been used to treat neurological diseases in a series of clinical trials [4,5]. It's widely believed that the protective

mechanisms attributed to MSCs involve the secretion of substances engaged in anti-inflammatory, proliferative and anti-apoptotic response. These cells can rapidly migrate to ischemic sites [6,7] and infiltrate in the brain parenchyma, which may interact primarily with microglia or endothelial cells [8], as well as neurons and astrocytes [9,10].

Experimental models of epilepsy have been developed as an attempt to expand knowledge of human epilepsy and develop more effective treatments. The maximum electroshock-induced seizure (MES) is a classical experimental model to induce generalized tonic-clonic seizures in rodents. This model mimics seizures commonly found in drug-resistant patients, and is the chosen model for primary screening of new antiepileptic drugs [11,12]. The seizure induced by

Citation: Tamura BP, Almeida DC, Felizardo RJ, Olanda GC, Bocca LF, et al. (2014) Convulsive Seizure Protection after Hippocampal Transplantation of Mesenchymal Cells from Adipose Tissue in Mice. J Stem Cell Res Ther 4: 196. doi:10.4172/2157-7633.1000196

MES generates a tonic flexion and extension behavior of the upper and / or lower limbs, followed by clonus. After the clonic and tonic phases, the animal shows loss of postural reflex followed by a period of post-ictal depression whose duration depends on the intensity of stimulation [13]. The screening tests evaluate the treatment ability in preventing the seizure activity across the neuronal tissue, which is indicated by blocking or reducing mainly the tonic component of generalized seizures [14,15]. Interestingly, the induction of MES has been implicated in alterations in cytokines gene expression, which indicates a commitment of inflammatory responses to the convulsive stimulus [16,17].

Based on these evidences, we propose to test the hypothesis that mesenchymal cells from adipose tissue (MCAT) transplanted in the hippocampus may have anticonvulsant effects on the behavioral and immunomodulatory mechanisms leading to a protection against tonic seizures and reduction of animal mortality induced by the MES model and in the pro-inflammatory cytokine levels, as well as an induction of anti-inflammatory responses. Thus, this study investigated the protective effect offered by the MCAT cells against acute epileptic seizures.

Methods

Subjects

Subjects were C57BL/6 and transgenic C57BL/6 expressing EGFP (enhanced green fluorescent protein) male mice, aged 8-12 weeks at the beginning of the experiments. They were housed in a pathogen-free facility and maintained in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council), in groups of 3-5 in polypropylene cages (28 x 17 x 13 cm) with wood shavings on the floor, under standard laboratory conditions, having free access to food and water. Room temperature was kept at 21ºC ± 1ºC and lights (50 lux) were on at 07:00 h and off at 19:00 h. All protocols were approved by the Ethics Committee of the Universidade Federal de São Paulo (UNIFESP), process 145/10 under approval of the National Commission of Ethics in Research (CONEP/MS).

Isolation and preparation of mesenchymal stem cells from adipose tissue (MCAT)

MCAT cells were isolated from C57/B6 mice by enzymatic digestion (collagenase type 1A) of the supra-epididymal and subjected to the process of culture with DMEN Low (Dulbecco's Modified Eagle's Medium Low) supplemented with 10% fetal calf serum (FCS). The MCAT was characterized by morphology and multipotent potential conforming described in Bassi and co-authors [18]. Subsequently, the cells were expanded, characterized in vitro to confirm their mesenchymal nature, and transplanted when they reached approximately 80% confluence in culture.

Cell transplantation of the mesenchymal cells from adipose tissue (MCAT)

After grown in culture, MCAT were labeled with the fluorochrome PKH26 (Sigma Aldrich) by incubation for 30 min immediately before transplantation. Then, MCAT cells were bilaterally transplanted in mice (MCAT, n = 12) by stereotaxic surgery, 1×10^5 cells into the hippocampus (50,000 per hemisphere). The stereotaxic coordinates used for the MCAT transplantation have the bregma as a reference and these are: - 2.0 mm in the antero-posterior, -1.5 mm and +1.5 mm in medial-lateral for both sides, and -2.5 mm dorso-ventral

directions [19]. The cell infusion was performed by injecting cells in culture medium suspension with a Hamilton syringe (10 μL) at a rate of 1uL/15 seconds in order to avoid embolism by mesenchymal cells in the hippocampus or adjacent structures. The injection canullae was removed one minute after the inoculation. The same procedure was performed for the control group (CTRL, n=12), injecting the same volume of culture medium in the same points.

Convulsive seizure induction by maximum electroshock seizure model (MES)

Between seven and ten days after the transplantation surgery, the group of transplanted mice (MCAT-MES group, n=12) and the group that received only the culture medium (CTRL-MES group, n=12) underwent maximum electroshock seizure (MES), which is an electroshock stimulus (65 mA, 60 Hz, 0.15 s duration) applied via transcorneal to induced the tonic-clonic seizure. The procedure was performed for 5 consecutive days, at the same time of the day, due to their circadian cycles. The evaluated parameters to verify a possible protection against seizures were visual monitored and quantified for the presence or absence of individual components of the tonic-clonic frequency, the duration of each phase, including the post-ictal phase, and the mortality rate. The pattern and frequency of seizures were evaluated and quantified according to the scale of Racine for classification of seizures (1972) and compared between groups. We evaluated the occurrence, duration and intensity of each tonic/clonic component of seizure [12]. The parameters considered to estimate the anticonvulsant activity of the cells were the protection against leg extension and reduction of the duration of tonic seizure [11].

Immunohistochemistry

Five days after the MES, the animals were deeply anesthetized with 10% chloral hydrate and transcardiacally perfused with 50 mL of phosphate buffered saline (PBS) followed by 250 mL of 4% paraformaldehyde. The brains were dissected and coronal brain cryostat sections (35-μm thick) were made between bregma +1.10 and -3.08 mm, according to the stereotaxic coordinates of the mouse brain atlas [15]. Because MCAT cells were already labeled with fluorochrome PKH26, the cells were identified by fluorescence microscopy directly under the Nikon 80i, filter (red), and it was not used any specific antibody to visualize the transplanted cells in the hippocampus. The slices were incubated with three distinct markers, chosen to verify the phenotype of neurons (NeuN), astrocytes (GFAP) and microglia (Iba1), and used separately. The NeuN (1:2000), GFAP (1:2000) and Iba1 primary antibodies were made in rabbit, and all were combined with a secondary anti-rabbit Alexa-fluor488. All of the slides stained with these markers were examined using a fluorescence microscopy (Nikon 80i). In each section, double-labeled cells were searched for the presence of MCAT cells expressing these specific markers and their localization in the hippocampus. Sections were mounted on slides using a nuclear-counterstaining, fluorescence-preserving mounting medium containing DAPI (Vector).

For DCX immunohistochemistry, free-floating sections were incubated overnight with the primary antibody DCX (1:500) diluted in PBS. After incubation, the sections were washed in PBS and incubated with the ABC kit solutions (Vectashield, Vector, Burlingame, CA, USA) for 1.5 h. The sections were stained with diaminobenzidine (DAB, Sigma-Aldrich Corporation, St. Louis, USA) and mounted on gelatin-coated slides and coverslipped. Images were captured and digitized using the Nikon ACT-1 v.2 system.

Histological analysis

The qualitative analysis was performed by immunofluorescence for Iba1^{+} cells for morphological characteristics of microglia. The quantification of DCX positive cells was performed by light microscopy (Nikon 80i). The images were captured and digitised using the Nikon ACT-1 v.2 system and analysed with the Image J software. Four dorsal hippocampal slices per animal (4 slices for each marker) and an average of eight non-overlapping fields per slice, totalling 32 fields per hippocampus for each animal were analysed at 40x magnification. In each section, nuclear profiles of DCX were counted by an observer blind to the experimental condition.

Gene expression

Fresh hippocampal samples were collected to quantify the expression of IL-1β, IL-6, IL-4, IL-10, TNF-α, caspase-1 and iNOS genes. Total RNA was extracted using TRIzol® reagent (Life Technologies, USA), and isopropanol precipitation. After RNA isolation, the concentration was made using a Nanodrop spectrophotometer (Thermo Scientific, USA). The mRNA was reversely transcribed using the High Capacity kit (Life Technologies, USA), and primed with Oligo dT. The expression level of each evaluated gene was measured by qPCR using the TaqMan system (Life Technologies, USA) and the equipment ABI Prism 7300 (Life Technologies, USA). The endogenous HPRT gene was used to normalization. The amplification program was as it follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 30 seconds and 60°C for 1 minute. The results were analyzed through the URE method of relative quantification (10,000/2^ΔCt) using the SDS software (Life Technologies, USA).

Statistics

Statistical analyses were performed using Prism software (version 288 5.01, GraphPad Software, San Diego, CA, USA). Convulsive seizure behavior, cell quantification and PCR gene expression were analyzed with Student t-test Data are presented as the mean ± S.E.M. Differences were considered significant when $p<0.05$.

Results

Behavioral seizure analysis

In order to verify the potential of modulation of these cells in repetitive intense stimulus, the seizures induced by MES were applied starting 10 days after transplantation of MCAT or DMEM (CTRL) during 5 consecutive days (named as day 1, day 2, day 3, day 4, day 5). The seizures were analyzed in each day from the 1st to the 5th day comparing both groups, CTRL-MES and MCAT-MES, for the frequency and duration of each phase, including the post-ictal phase, and the total mortality rate of the 5 days.

All animals (100%) presented clonic seizures in all of the 5 days. At the 1st day, 90% of CTRL-MES and 100% of MCAT-MES groups presented tonic seizures. At day 2, 85% of CTRL and 100% of MCAT groups presented tonic seizures. In the subsequent days (day 3, 4 and 5), 100% from both CTRL-MES and MCAT-MES presented tonic seizures. When comparing these data, no difference was found in the frequency of animals presenting seizures between the two groups.

The analysis of the duration (sec) of tonic seizure showed a decrease of time period on the MCAT transplanted animals on day 3, day 4 and day 5 compared with CTRL-MES group (respectively p=0.0045; p=0.0472; p=0.0272), indicating a long-term protective

action of MCAT-MES, with an initial delay for this modulation (Figure 1A-1C). Additionally, it was observed a significant reduction in the duration of the post-ictal phase in the MCAT transplanted group (p=0.0128), indicating faster recovery after the convulsion, which is also an indicative of protection. The duration of clonic seizures was significantly increased in MCAT-MES when compared to the CTRL-MES group (5 days average p=0.0007 and p<0.01 for each day; Figures 1D and 1E) suggesting that the generalized seizure had to manifest itself somehow, but protection of tonic phase induced increased in seizure manifested as clonia. The differences between the times of tonic and clonic seizures within the same group implied that the total time of generalized seizures was not different between the groups.

Interestingly, the mortality rate was reduced in the MCAT-MES transplanted animals when compared with CTRL-MES group (Chi-square p<0.0001). The protection in mortality of MCAT animals was probably related to the reduction in time of tonic seizures observed in this group (Figure 1F).

Hippocampal immunochemistry

A qualitative analysis in the host hippocampus of animals transplanted with MCAT cells was performed by immunofluorescence to confirm the presence, localization into the hippocampal regions and expression of neuronal, astrocyte and microglial markers (NeuN, GFAP and Iba1). The presence of MCAT cells in the hippocampus was confirmed in all transplanted animals and most of the MCAT cells were found preferentially at dentate gyrus (DG). This finding indicated that MCAT transplanted cells had survived after the application of MES for 5 days. The MCAT cells were detected in clusters, closely associated to the site of injection (DG), and also found scattered in the path of the needle (Figures 2A and 2B).

Some MCAT cells double-stained for Iba1 marker were detected, and high concentration of resident microglial expressing Iba1 was present at the site of injection (DG), surrounding the MCAT transplanted cells (Figures 3A-3C). No MCAT cells expressing NeuN and GFAP markers were detected in the hippocampus (Figures 3D-3I). Quantification of DCX positive cells was done in the hippocampus, and did not differ between CTRL-MES and MCAT-MES groups (Figures 4A-4C).

Cytokine gene expression analysis

To investigate the immunomodulatory mechanisms by which the MCAT therapy resulted in tonic seizure protection, we tested whether the transplantation of MCAT cells modulates the hippocampal gene expression of Il-1β, IL-6, IL-4, IL-10, iNOS, caspase-1 and TNF-α. The results indicated that the levels of IL-1β and IL-6 in MCAT-MES group decreased compared to CTRL-MES (Student t test, p=0.0266 and p=0.0206, Figures 5A and 5B). In addition, the transplantation of MCAT cells also reduced levels of iNOS and caspase-1 in the hippocampus (p=0.0405 and p=0.0034, Figures 5C and 5D). The anti-inflammatory cytokine IL-4, we detected increased in MCAT-MES group than CTRL-MES (p=0.0025, Figure 5E). The levels of IL-10 and TNF-α in the hippocampus did not differ significantly between the groups (data not shown).

Discussion

Our findings indicated that MCAT cells transplanted into the hippocampus exert anticonvulsant effects by blocking the tonic component of generalized seizures and reducing the mortality rate
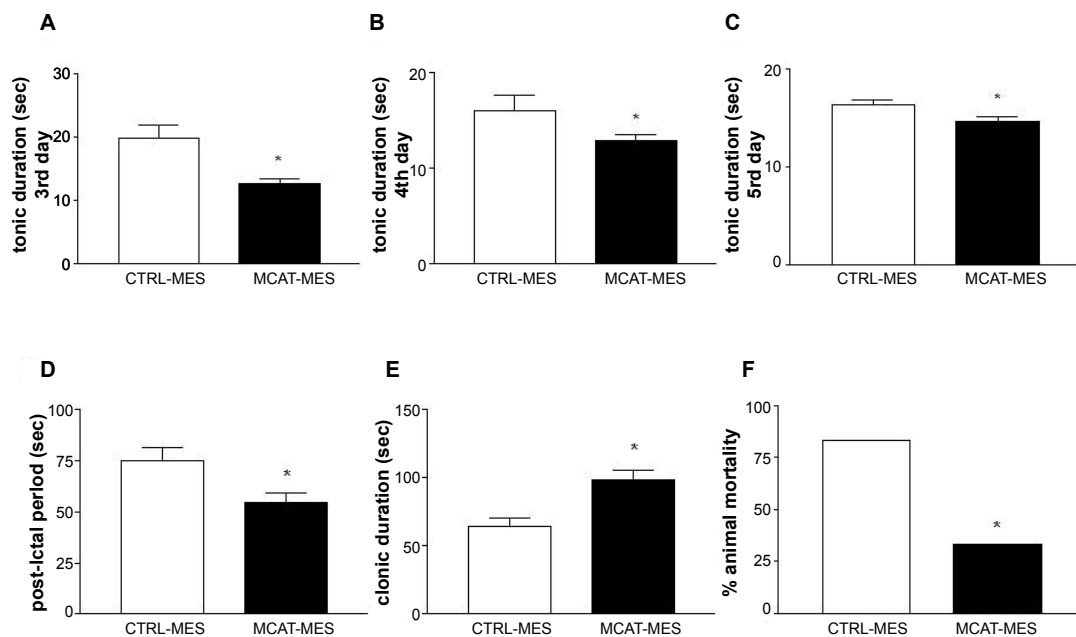
Figure 1: Evaluated parameters in seizures induced by MES. Analysis of tonic duration indicated a reduction in day 3 (A), day 4 (B) and day 5 (C) of MES stimulation for the MCAT-MES group (n=12) when compared to the CTRL-MES group (n=12); D) The duration of post-ictal period also decreased in MCAT-MES group; E) Clonic seizures after MES stimulation comparing CTRL-MES and MCAT-MES groups indicated an increase in MCAT-MES; and F) percentage of mortality rate shows considerable reduction in of MCAT-MES group compared to CTRL-MES group (*$p < 0.05$, Student t test; in F Chi-square). From A to E, data represented by mean $\pm$ S.E.P.
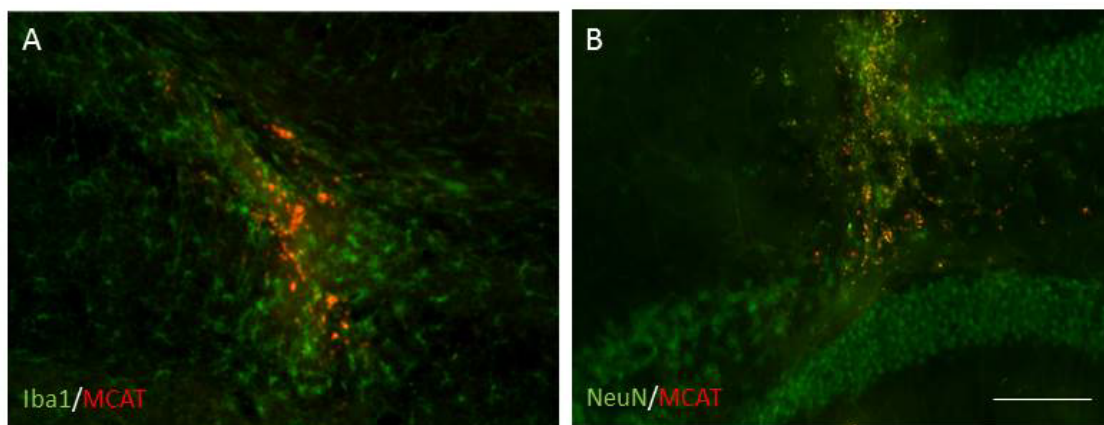

Figure 2: Images from fluorescence microscopy showing MCAT cells stained with fluorochrome PKH26 in the hippocampus. A) MCAT cells survived after the application of MES for 5 days and were detected in clusters in MCAT-MES transplanted mice; B) MCAT cells close to the site of injection (DG), and also found scattered in the path of the needle MCAT-MES. Scale bar 100 µm.

induced by MES. In addition, the seizures in MCAT-transplanted mice triggered an acute inflammatory response, changing the pattern of IL1-β, IL-6, iNOS, caspase-1 and IL-4 gene expression. The immunofluorescence analyses confirmed the presence of MCAT cells in the hippocampus of transplanted animals and the co-localization of MCAT cells and neural marker was not detected. The immunomodulatory and anti-inflammatory properties of mesenchymal cells are well known and have been emerging as a new therapeutic alternative for a wide variety of degenerative and immune disorders. Based on our findings, we questioned whether the MCAT cells, when transplanted into the hippocampus, could have a local

action that would influence the neuronal activity in the hippocampus by modulating the excessive and synchronous neuronal firings that result in seizures, or acting by releasing modulatory substances which could "turn off" the tonic activity of generalized seizures.

Browning and colleagues suggested that generalized seizures have distinct neuroanatomical substrates that start and sustain the seizure activity [20]. It is possible that the site of injection of the mesenchymal cells (hippocampus) has influenced the weakening of convulsive behavior of transplanted animals. The hippocampus, as already well described, plays an important role in triggering and amplifying
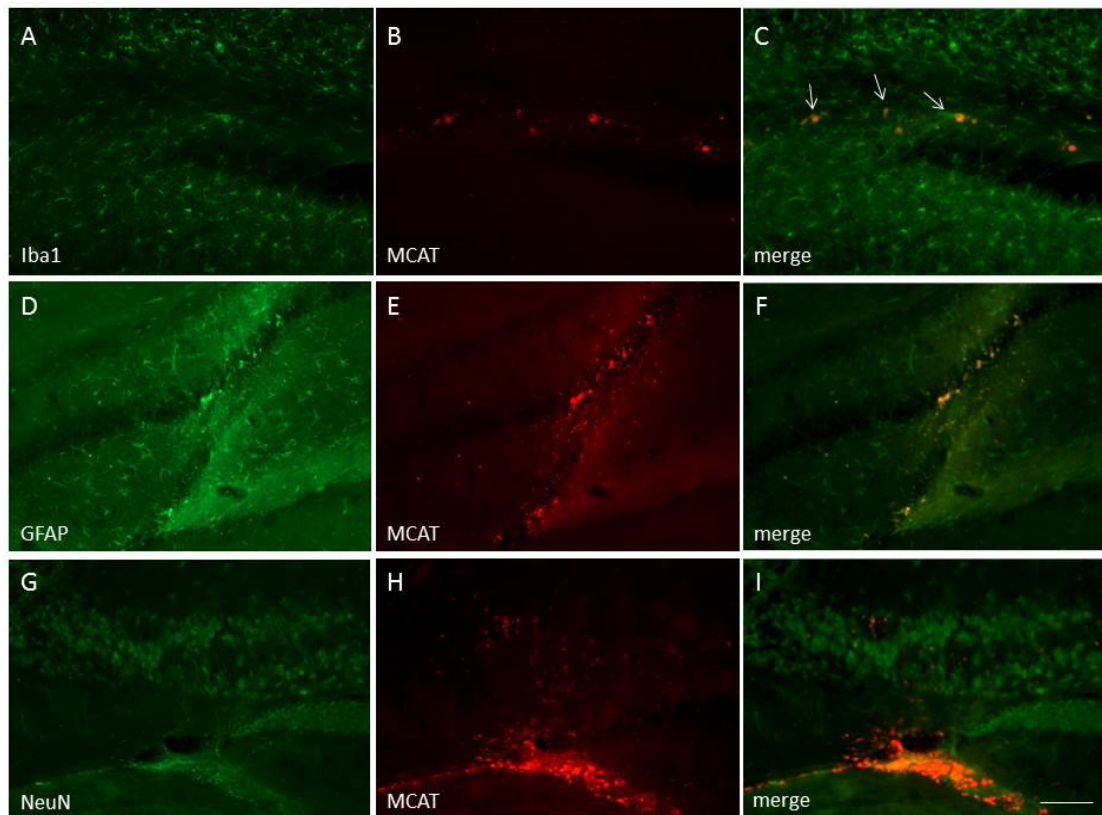
Figure 3: MCAT cells (red) and NeuN, GFAP and Iba1 markers (green) in the hippocampus. A) Iba1+ cells (green) present at the site of injection (DG), surrounding the MCAT transplanted cells (B); In C) note the microglia phenotype of MCAT cells (arrows) in the dentate gyrus (DG). D-I) No co-localization of MCAT cells (red) with GFAP and NeuN markers (green) in the hippocampus. Scale bar 100 μm.
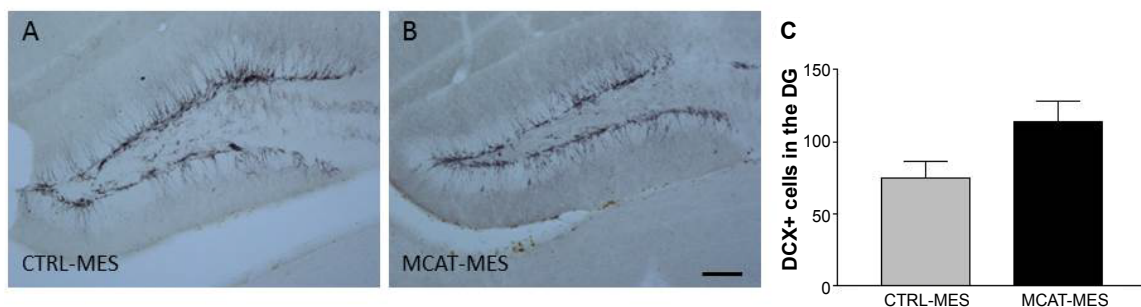

Figure 4: Doublecortin (DCX) images and quantification in the hippocampus of CTRL-MES and MCAT-MES groups. A) DCX in the dentate gyrus (DG) of a CTRL-MES mouse and B) MCAT-MES mouse; in C) no difference was found in the number of DCX+ cells counted in the DG between CTRL-MES and MCAT-MES groups. In A and B scale bar 50 μm. In C data represented by mean ± S.E.P.

epileptic seizures. This structure might lose or diminish the potential of inducing seizures, since the mesenchymal cells have been injected into the dentate gyrus. In this case, one possible reason to explain the protection effect is that the transplanted cells showed a barrier effect, preventing the hyperexcitability in the local circuit and avoiding the spread (generalization) of seizures, expressed by reduction in duration (tonic) and in post-ictal period. Interestingly, in these transplanted animals the duration of clonic seizures increased as a compensatory effect of the inhibition of tonic seizure, as if the generalized seizure had to manifest itself somehow.

The hippocampus, a structure susceptible to convulsive seizures [21,22] contains cells vulnerable to electroshock [23-25]. In fact, the stimulus of the electrochock, regardless the MCAT treatment, induced an increased in the hippocampal neurogenesis and neuronal death. The presence of MCAT cells in the hippocampus could just be a mechanism induced by the cells to replace cells in this area (known for its susceptibility to neuronal loss), rather than a direct effect to protect seizures. However, MCAT cells were not co-localized with neural markers and quantification of DCX did not differ between S-MES and MCAT-MES animals.
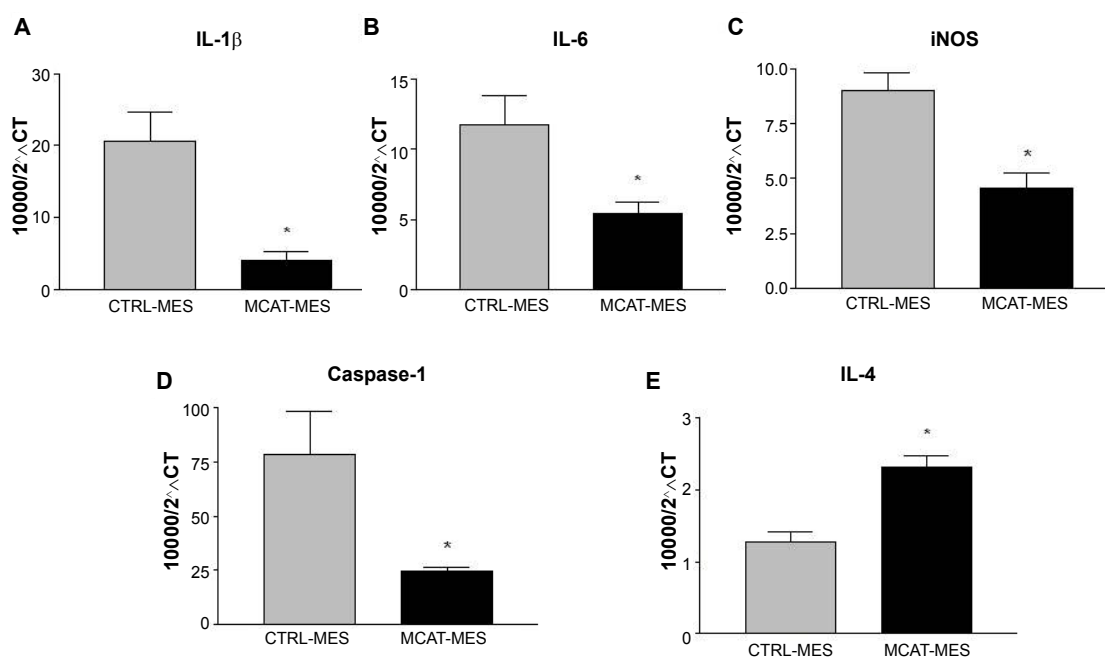
Figure 5: Hippocampal gene expression of Il-1β, IL-6, iNOS, caspase-1 and IL-4. In A) IL-1β; in B) IL-6; in C) iNOS and in D) caspase-1 dosed in the hippocampus of MCAT-MES group showed a decreased compared to CTRL-MES; in E) IL-4 showed an increased in MCAT-MES group compared to CTRL-MES (*p< 0.05, Student t test). Data represented by mean ± S.E.P.

As already described, microglial cells present morphological alterations after maximum electroconvulsive shock (MES) [16,17,26]. These authors described a reduction in the density of microglial processes after MES, which is indicative of an intermediate stage between inactive and reactive microglia [27,28]. We noticed that part of the MCAT population expressed the microglial marker Iba1 and developed microglia phenotype. Also, our results corroborate others that described an increase in Iba1+ cells around the transplanted mesenchymal cells in the hippocampus of rats with Alzheimer's disease [29]. As proposed by Silverberg and co-authors, inflammatory cells are recruited to regions of the brain associated with the epileptic focus [30]. These authors suggested that in MES-induced seizures, electric currents spread throughout the brain and the intensity and/or duration of stimulation can change the levels of cytokines and chemokines. Nevertheless, is still unclear whether Iba1+ cells present in the hippocampus play a role on generalized or focal seizures and/or directly participate on clonic seizures that persist.

As suggested by Jankowski and Patterson [28], the beginning and duration of seizure are influenced by a variety of types and levels of cytokines that, at the time of induction, can compete to determine the behavioral outcome. Several studies have reported the high concentration of IL-6 immediately after the seizure [31,32]. Moreover, the proconvulsant effects of IL-1 β have been proposed since its levels in the brain increased during seizures [33]. Interestingly, the inhibition caspase-1, which is involved in the production of the active form of IL-1β and plays a role reducing the brain levels of IL-1 β, has been suggested as an effective anticonvulsive strategy [34]. In our experiment, the gene expression of IL1-β, IL-6, caspase-1 and iNOS was significantly lower in animals transplanted with MCAT. This reduction in pro-inflammatory cytokines as well as caspase-1 and iNOS can be related to the reduction of tonic seizure in the MCAT-MES group. Together with

this reduction, the expression of anti-inflamatory cytokine IL-4 was increased in the MCAT transplanted animals, which may have reduced the susceptibility to seizure-induced pro-inflammatory cytokines. Although increase in IL-10 was not detected, our data corroborate other findings that showed a reduction of pro-inflammatory and increase of anti-inflammatory cytokines in chronic epileptic rats treated with bone marrow cells [35]. Similarly, other authors proposed that the expression of IL-10 can be induced simultaneously with pro-inflammatory cytokines in brain after an insult [36,37]. The beneficial or harmful role of the innate immune response in epileptic tissue, however, still needs to be clarified [38].

Taken together, our results suggest that the protective effects of MCAT cells are possibly promoted by the action of inhibitory factors and immunomodulatory mechanisms assigned to mesenchymal cells on seizure spreading. Moreover, we suggest that the anticonvulsant potential of MCAT cells can be tested by MES model, using the same paradigm used in pharmacological screening tests of drugs. Taking this contribution into account, several considerations concerning the MCAT cells, neuro-regulatory molecules and cytokines secretion, as well as growth factors that mediate the protective effects on convulsive seizure should be elucidated by further experiments. This study brings therapeutic implications for the control of epileptic seizures and may open interesting discussions on the mechanisms relevant to the pathophysiology of epilepsy.

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