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Pericytes Extend Survival of ALS SOD1 Mice and Induce the Expression of Antioxidant Enzymes in the Murine Model and in IPSCs Derived Neuronal Cells from an ALS Patient

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Abstract Amyotrophic Lateral Sclerosis (ALS) is one of the most common adult-onset motor neuron disease causing a progressive, rapid and irreversible degeneration of motor neurons in the cortex, brain stem and spinal cord. No effective treatment is available and cell therapy clinical trials are currently being tested in ALS affected patients. It is well known that in ALS patients, approximately 50% of pericytes from the spinal cord barrier are lost. In the central nervous system, pericytes act in the formation and maintenance of the blood-brain barrier, a natural defense that slows the progression of symptoms in neurodegenerative diseases. Here we evaluated, for the first time, the therapeutic effect of human pericytes in vivo in SOD1 mice and in vitro in motor neurons and other neuronal cells derived from one ALS patient. Pericytes and mesenchymal stromal cells (MSCs) were derived from the same adipose tissue sample and were administered to SOD1 mice intraperitoneally. The effect of the two treatments was compared. Treatment with pericytes extended significantly animals survival in SOD1 males, but not in females that usually have a milder phenotype with higher survival rates. No significant differences were observed in the survival of mice treated with MSCs. Gene expression analysis in brain and

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spinal cord of end-stage animals showed that treatment with pericytes can stimulate the host antioxidant system. Additionally, pericytes induced the expression of *SOD1* and *CAT* in motor neurons and other neuronal cells derived from one ALS patient carrying a mutation in *FUS*. Overall, treatment with pericytes was more effective than treatment with MSCs. Our results encourage further investigations and suggest that pericytes may be a good option for ALS treatment in the future.

Keywords Amyotrophic lateral sclerosis · Pericytes · Mesenchymal stromal cells · SOD1 mice · IPSCs · Motor neurons

Abbreviations

ACTB	Beta Actin
ALS	Amyotrophic Lateral Sclerosis
basic FGF	Basic fibroblast; growth factor
BBB	Blood Brain Barrier
BNDF	Brain-derived Neurotrophic Factor
BSA	Bovine serum albumin
CAT	catalase
CD11b	cluster of differentiation; molecule 11B
CFDA-SE	carboxyfluorescein diacetate succinimidyl ester
CHAT	Choline acetyltransferase
c-Myc	Proto-oncogene c-Myc
DAPI	4′,6-Diamidino-2-phenylindole dihydrochloride
DMEM	Dulbecco's Modified Eagle Medium
EB	Embryoid bodies
EBM	Endothelial Cell Growth Medium
EGF	Epidermal growth factor
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factors
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GDNF	glial cell derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GLP-1	Glucagon-like Peptide 1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPX	glutathione peroxidase
GSR	Glutathione reductase
HBSS	Hanks' Balanced Salt Solution
IFN-g	Interferon gamma
IGF-1	Insulin-like growth factor 1
IL-10	Interleukin 10
IL-12p70	Interleukin 12p70
IL-13	Interleukin 13
IL-15	Interleukin 15
IL-17A	Interleukin 17A
IL-1ra	Interleukin 1ra
IL-1β	Interleukin 1β
IL-2	Interleukin
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-7	Interleukin 7
IL-8	Interleukin 8
IL-9	Interleukin 9
IP-10	C-X-C motif chemokine 10
IPSC	Induced Pluripotent Stem Cells
Klf4	Kruppel-like factor 4
MCP-1	Monocyte chemoattractant protein-1
MIP-1α	Macrophage Inflammatory Proteins −1α
MIP-1β	Macrophage Inflammatory Proteins -1 β
MND	Motor neuron diseases
MNX1/HB9	Motor neuron and pancreas homeobox 1/ Homeobox HB9
MSCs	Mesenchymal stromal cells
NB media	Neuro Basal Media
NF-kB	factor nuclear kappa B
NGF	Nerve Growth Factor
NPCs	neural progenitor cells
NSCs	Neural Stem Cells
Oct4	octamer-binding transcription factor 4
PaGE	paw grip endurance
PBS	Phosphate-buffered saline
PDGF –BB	Platelet-derived growth factor C

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RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
Ri	ROCK inhibitor
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
SHH	Sonic Hedgehog
SOD1	superoxide dismutase 1
Sox2	SRY (sex determining region Y)-box 2
TNF-α	Tumor necrosis fator α
VEGF	Vascular endothelial growth factor

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a devastating late onset neurodegenerative disease [1]. Both upper and lower motor neurons can be affected, leading to an irreversible degeneration [2].

Approximately 90% of all ALS cases are sporadic (SALS) and the remaining 10% comprises the familial forms (FALS) [3]. Mutations in *SOD1,* which encodes the antioxidant enzyme superoxide dismutase 1, represent one of the most frequent causes of FALS. Mutated *SOD1* can form intracellular aggregates in a trimeric form that interfere in motor neuron survival [4].

The only drug approved by the FDA for ALS is riluzole, which increases only 2 to 4 months patients' survival [5–7]. Cell therapy is amongst the therapies being tested for this disease, with clinical trials currently being conducted in humans (NCT02017912 and NCT01730716). However, some important issues such as determining the effectiveness of different cell sources, injection protocol and the best immunosuppressive regimen, still need to be established [8].

Pericytes are CD146+ perivascular cells being considered for treatment of different pathologies [9–12]. They possess common features with MSCs such as, the expression of determined surface markers, secretion of diverse paracrine factors and the ability to differentiate [13, 14]. Evidences indicate that MSCs are derived from these perivascular cells, that are liberated from their basement membrane surrounding blood vessels upon injury or inflammation [15].

Within the diverse tissues, pericytes play important cellular functions such as vascular stability and angioarchitecture. In the central nervous system, pericytes act in the formation and maintenance of the blood-brain barrier (BBB) and consequently help to protect the neuronal environment [16]. In neurodegenerative diseases, the BBB breakdown allows blood components to infiltrate the neuronal environment, aggravating the existing inflammation and accelerating the progression of symptoms [16, 17]. In ALS patients, approximately 50% of pericytes from the spinal cord barrier (BHC) are lost [18].

Here we evaluated the effect of pericytes as compared to mesenchymal stromal cells (MSCs) in vivo in SOD1 mice and in vitro in human ALS motor neurons and other neuronal cells.

Materials and Methods

All experiments were carried out in the Human Genome and Stem Cell Research Center, at the Biosciences Institute, University of São Paulo. The use of human MSCs and pericytes in this study was approved by the Biosciences Institute Ethics Committee on Human Subject (Comitê de Ética em Pesquisa do Instituto de Biociências da Universidade de São Paulo – IBUSP #5464/ Certificate CAAE # 20108413.4.0000.5464) and informed consent was obtained from the healthy donor.

Animal experiments were approved by the local animal care committee (Comissão de ética no uso de animais do IB USP/ Certificate #2013.1.1198.41.0) and were in compliance with the Guidelines of the Brazilian College of Animal Experimentation (COBEA) and NIH Guide for Care and Use of Laboratory Animals.

Cell Preparation

The adipose tissue sample was obtained from one healthy female donor that underwent total hysterectomy, after informed consent. The tissue sample was processed according to a protocol described elsewhere [10, 14]. Briefly, the sample was washed extensively with PBS, minced with scalpels and incubated with 1 mg/mL of collagenase type II (Sigma Aldrich) in a 1:3 ratio (tissue weight: collagenase volume) diluted in DMEM-F12 supplemented with 20% fetal bovine serum (FBS), 1% non-essential amino acids (GIBCO) and 1% antibiotics/antimycotics (GIBCO) and incubated for 30–40 min in a shaker, at 250 RPM and 37 °C. Cells were then passed through a 70 μm strainer and incubated in blood lysis solution for 5–10 min. PBS was added in a 2:1 ratio and the solution was filtered again through a 40 μm strainer. In this step, the sample was divided into two parts: one corresponding to mesenchymal stromal cells, immediately plated and the other corresponding to the pericytes, prepared for cell sorting. For that purpose, cells were incubated with conjugated antibodies for CD-34 (Percp-Cy5.5), CD-45 (FITC), CD-56 (APC) and CD 146 (PE), all from BD Biosciences. DAPI was added just before the analysis, and all cells positive for DAPI were excluded (dead cells). Pericytes (CD146+/CD34−/ CD45−/CD56-) were sorted into a 24 well plate at a density of 20.000 cells/cm^2.

Pericytes and MSCs were cultured and trypsinized after they reached 80% confluence. For cell passaging, a density of 5000 cells per cm^2 was considered. MSCs were cultured in DMEM-F12 medium containing 1% Glutamax (Gibco), 20% FBS (Gibco), 1% non-essential amino acids (Gibco) and 1% antibiotics/antimycotics (Gibco). Pericytes were cultured in EBM-2 medium (Lonza).

For in vitro and in vivo experiments, cells in 8th passage were used. The expansion of cells was necessary in order to

obtain an ideal amount of cells that were subsequently used in all experiments. The secretome of MSCs varies according to the niche from which MSCs were obtained and from different donors [19]. To avoid this variability in our results, we used the same lineage of MSCs and pericytes for all experiments. Importantly, these lineages were obtained from the same healthy donor.

Cell Line Characterization

MSCs and pericytes were evaluated according to their in vitro potential of differentiation into adipocytes, chondrocytes and osteoblasts using Stem Pro differentiation kits (Invitrogen) according to the manufacturer's instruction. Surface markers were evaluated by flow cytometry. In addition we performed MLPA analysis to attest the integrity of the DNA in subtelomeric regions prior the experiments. These evaluations were performed at 8th passage, the same used for all experiments.

For chondrogenic differentiation, cells were resuspended in PBS and droplets of 5 μL were dripped in a 24-well plate, spaced from each other (concentration ranging from 10^5 to 10^7 cells per drop of 5 μL). After incubation for 2 h at 37 °C, growth medium or differentiation medium were gently added in control wells or experimental wells, respectively. Medium was changed every 3–4 days, until achieving 21 days of culture, when differentiation was analyzed by the presence of extracellular matrix mucopolysaccharides. For that purpose, cells were washed with PBS and fixed with paraformaldehyde 4% for 30 min. Toluidine 1% was added and cells were incubated for 30 min.

For adipogenic differentiation, cells were cultured in a 24-well plate, until reaching 80% confluence. Growth medium was added in control wells and differentiation medium, in experimental wells. Medium was changed every 3–4 days, until achieving 21 days of culture, when differentiation was evaluated by intracellular accumulation of lipid-rich vacuoles, stained with Oil red O (Sigma-Aldrich). Cells were fixed with 4% paraformaldehyde for 30 min, washed, and stained with a working solution of 0.16% Oil red O for 20 min.

For osteogenic differentiation, cells were cultured in a 24-well plate, until reaching 80% confluence. Growth medium was added in control wells and differentiation medium, in experimental wells. Medium was changed every 3–4 days, until achieving 21 days of culture, when differentiation was evaluated by accumulation of mineralized calcium phosphate assessed by Alizarin Red staining (Sigma-Aldrich). Cells were fixed with 70% ethanol for 30 min, washed with deionized H_2O, and stained with Alizarin Red for 30 min.

To analyze cell surface expression of typical protein markers, adherent cells were pelleted, resuspended in PBS at a concentration of 1×10^5 cells/mL and stained with saturating concentration of the following anti-human antibodies: CD29-PECy5, CD34-PerCP, CD31-PE, CD45-FITC, CD90-PE, CD117-PE, human leukocyte antigen (HLA)-ABC-FITC, HLA-DR-PE (Becton, Dickinson and Company), and CD 146. Cells were incubated in the dark for 45 min at room temperature. After incubation, cells were washed with PBS and resuspended for analysis. A total of 10,000 events were analyzed using a Guava EasyCyte flow cytometer running Guava Express Plus software (Guava Technologies).

Multiplex ligation-dependent probe amplification (MLPA) analysis was performed using SALSA P070 and SALSA P036 subtelomeric probes (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions, to confirm the integrity of the lineages before starting the experiments.

These kits contain one probe for each of the 41 subtelomeric regions and 5 probes near the centromeric regions of the five acrocentric chromosomes. P070 probes differ from the P036 probes.

Lymphocyte Proliferation Assay

The lymphocyte proliferation assay was performed to assess the immunomodulatory potential of MSCs and pericytes. Increasing concentrations of MSCs and pericytes were cultured in a 96-well plate (10^3, 5.10^3, 1.10^4, 5.10^4, 1.10^5 cells per well). After attachment, cells were treated with mitomycin C (Sigma) for 3 h to inactivate cell cycle. Subsequently the cells were washed twice with PBS.

Peripheral blood mononuclear cells (PBMCs) were collected from peripheral blood of one symptomatic SOD1 female and separated from erythrocytes by a Ficoll-Paque (GE) gradient, according to the manufacturer's instructions. PBMCs were labeled with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Invitrogen) and were seeded at a constant concentration of 1.10^4 cells in each well, in the same plate containing inactivated MSCs or pericytes. All experiments were carried out in triplicates.

Anti-mouse-CD3 antibody was added to the culture medium to stimulate mouse lymphocyte proliferation. The assay was performed in triplicates and activated lymphocytes cultured alone served as control. Cells were maintained in RPMI medium at 37 °C and 5% CO_2 for 5 days. Thereafter, the lymphocytes were resuspended and cell division fluorescent peaks, obtained by the dilution of CFDA staining in the cytoplasm of daughter cells, were evaluated. A total of 10,000 events were analyzed using a Guava EasyCyte flow cytometer running Guava Express Pro software (Guava Technologies). The experiment was carried out in triplicates. For analysis, mean fluorescence was used.

Macrophage Phagocytosis Assay

This assay was conducted in order to assess the potential of macrophages, obtained from SOD1 mice, to phagocyte

human MSCs or pericytes. Macrophages were obtained after peritoneal cavity wash from one symptomatic female SOD1, according to a previously described protocol [20]. After washing with PBS, cells were centrifuged (400G, 5 min) and plated (2.10^5 cells per each well, in a 24-well plate) in RPMI medium with 10% FBS. In the following day, non-adherent cells were removed, leaving only the macrophage population. 2.10^4 MSCs and pericytes were plated in macrophage containing plate. The experiment was carried out in triplicates.

Cells were maintained at 37 °C and 5% CO2 for 72 h. After this period, immunofluorescence was conducted to allow human cells (MSCs or pericytes) counting. For this purpose, after fixation with 4% paraformaldehyde for 20 min, cells were washed and permeabilized for 15 min in 0.2% Triton-X and 1% BSA, diluted in PBS. Blocking was performed for 1 h with 5% BSA, 10% FBS, 0.1% Triton-X, followed by incubation with human specific primary antibody anti-lamin A/C – (Vector, 1: 100), overnight at 4 °C. After washing with PBS, secondary antibody (donkey anti-mouse Alexa 488, 1: 400) was added and incubated for 1 h. In the last 15 min of incubation, DAPI was added. Human cells (labeled with lamin A/C and DAPI) and macrophages (DAPI) were analyzed with a fluorescence microscope and quantified with ImageJ.

Animal Model and Experimental Groups

SOD1 mice harboring G93A mutation with B6SJL background were purchased from the Jackson Laboratory (Bar Harbor, ME http://www.jax.org) and bred in collaboration with IPEN (Instituto de Pesquisas Energéticas e Nucleares), to form our colony. Animal care and experiments were performed in accordance with the animal research ethics committee of the Biosciences Institute, University of São Paulo.

Among the different animal models for ALS, SOD1 mice are the most used in preclinical studies. After the initial tremor in the limbs they develop muscle weakness in early adulthood, become fully paralyzed and die [21–23]. These mice overexpress the human SOD1 gene bearing the G93A mutation, a point mutation found in familial ALS. Interestingly, in this animal model the disease progression is different between the genders. Males have a shorter lifespan and a clinical condition apparently more severe than females and differences in electrophysiological parameters have also been reported [24, 25]. Interestingly, a comparable influence of gender is also observed in ALS patients [26].

Eight week old mice were separated in three groups ($n = 22$ per group on average, including 11 females and 11 males). At this age, SOD1 mice are clinically considered asymptomatic but already present motor neuron abnormalities due to disease progression [23]. Animals from each group received weekly transplantation of either pericytes, MSCs or Hanks' Balanced Salt Solution (HBSS, vehicle). Each injection contained 1.10^6 viable cells in 100 μL HBSS or 100 μL HBSS alone and were delivered intraperitoneally until the animal's natural death or full paralysis (and therefore was considered as end-stage and sacrificed with ketamine overdose). This dose was decided based on our previous studies evaluating cell transplantation for different pathologies in mice (Secco et al., 2013; Valadares et al., 2014; Vieira et al., 2010; Zucconi et al., 2011).

For in vivo experiments, we followed the recommendations stated in a review of good practice for SOD1 mice studies [27]: (1) all data were carefully analyzed with specific statistical tests including the Cox mathematical model, (2) litter-matched gender-mice were used in each cohort, (3) a single uniform endpoint criterion was employed for each test, (4) tracking and exclusion of "non-ALS deaths" and long-lived animals.

In order to circumvent genetic background variations, pericytes were sorted from the total MSCs population from the same adipose tissue sample. We included 22 animals in each cohort since the initial amount of cells was sufficient to treat this number of animals *per* group. Statistical analysis was conducted accordingly.

Physical Performance Tests

All animals were assessed weekly from 8 weeks of age with a set of physical tests in order to determine whether injections of MSCs or pericytes influence the motor ability of treated mice. Besides weight evaluation, the tests included: motor score, paw grip endurance (PaGE) and rotarod, based on previously described protocols [28], with modifications.

For motor score mice were evaluated for signs of motor deficit using the following score: 5 points if normal (no sign of motor dysfunction), 4 points if slightly hind limb tremors are present when suspended by the tail, 3 points if hind limb tremors are evident when suspended by the tail, 2 points if gait abnormalities are present, 1 point for dragging of at least one hind limb, 0 points for inability to right itself within 30 s.

The rotarod test was used to evaluate motor coordination and fatigue resistance. For that purpose, the length of time each animal could remain on the rotating cylinder (3.5 cm) of a rotarod apparatus (IITC Life Science model 755) was recorded. The initial speed was 1 rpm and it was increased constantly until a final speed of 30 rpm, after 180 s. Each animal was given three tries and the longest latency to fall was recorded.

For the PaGE test, each mouse was placed on the wire-lid of a conventional housing cage. The lid was gently shaken to prompt the mouse to hold onto the grid before the lid was swiftly turned upside down. The latency until the mouse released at least both hind limbs was timed. Each mouse was given up to three attempts to hold on to the inverted lid for an arbitrary maximum of 90 s and the longest latency was recorded.

Cellular Reprogramming and Human Motor Neuron Differentiation

In order to evaluate the effect of MSCs or pericytes in a human-based experimental system, *we* used motor neurons and other neuronal cells (MNNs) derived from one ALS patient carrying a mutation in *FUS*. We chose to use a FUS lineage since it is well known that this protein, together with TDP-43, possess a possible pathogenic role in most types of ALS, except for SOD-1 linked ALS [29].

For human motor neuron differentiation, fibroblasts and derived IPSCs from one ALS male patient carrying a mutation in the *FUS* gene, were used after informed consent. Retrovirus vectors containing Oct4, c-Myc, Klf4 and Sox2 human cDNAs (obtained from Muotri's group) were used as described elsewhere [30]. Embryoid bodies (EBs) were formed by mechanical dissociation of cell clusters (pre-treated with dorsomorphin, 1 nM, for 2 days) and plated onto low-adherence dishes with NB media (DMEM/F12 plus 0.5X N2 and 0.5X B27 supplements) with dorsomorphin for 2 days and in the next 5 days with NB media containing FGF and EGF. After that, mature EBs were dissociated with accutase for 5 min at 37 °C and plated in matrigel with NB media plus FGF 20 ng/mL and EGF 20 ng/mL. Rosettes were visible for collection after 7 days and were then dissociated with accutase (Millipore) and plated onto poly-ornitine/laminin-coated dishes (Sigma) with NB media plus FGF and EGF. Homogeneous populations of neural progenitor cells (NPCs) appeared after 1–2 passages with accutase in same conditions.

NPCs were then dissociated with trypsin for 5 min at 37 °C and 4.10^4 cells were plated per well onto a 24 well plate. Neuronal differentiation was performed as described elsewhere [31, 32], and media was supplemented with BDNF (20 ng / mL), GDNF (20 ng / mL), IGF-1 (20 ng / mL), Ri (5uM), and SHH (100 ng / mL), for 3 weeks.

TaqMan Gene Expression Assay probes: *MNX1/HB9* (Hs00907365_m1), *CHAT* (Hs00252848_m1) and *ISL1* (Hs00158126_m1) were used to validate the derived MNNs. Human *ACTB* (β-actin; Hs01060665_g1) was used as a reference gene. RT-qPCR was performed in Applied Biosystems® 7500 Fast Real-time PCR system.

Co-Culture of MSCs or Pericytes with IPSCs Derived Motor Neurons and Other Neuronal Cells (MNNs) from an ALS Patient

After 3 weeks of neuronal differentiation, 2.10^4 MSCs or pericytes were plated onto 24-well 0,4 μM pore plate inserts (Millicell), to allow the passage of soluble factors only and avoid cell contact with human MNNs. Co-cultures were maintained for 7 days in NB medium supplemented with BDNF (20 ng/mL), GDNF (20 ng/mL), IGF-1 (20 ng/mL), Ri (5uM), and SHH (100 ng/mL). MNNs and MSCs or pericytes alone, under the same conditions were used as controls. Four replicas were made for each experimental condition. After 7 days of co-culture, the medium was removed and used for cytokine dosage. Total RNA was extracted from iPSCs derived cells and this was used for gene expression analysis.

Cytokine Quantification in the co-Culture Media of MSCs or Pericytes with Human IPSCs Derived Motor Neurons and Other Neuronal Cells (MNNs)

Cytokine levels were measured in Luminex 100 IS equipment (Bio-Rad) using BioPlex Pro Human Cytokine 27-Plex Kit, according to the manufacturer's instructions. The following pro- and anti-inflammatory cytokines were evaluated: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, eotaxin, basic FGF, G-CSF, GM-CSF, IFN-g, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF - BB, RANTES, TNF-α and VEGF. Four replicas were made for each experimental condition.

RNA Extraction and Gene Expression by Real-Time PCR for "In Vitro" and "In Vivo" Experiments

Gene expression analysis was performed with animals and with IPSCs derived cells. For in vivo experiments, two males and two females from each experimental group were sacrificed under deep anesthesia when animals were fully paralyzed (and therefore were considered as end-stage). Brain and spinal cord were collected and preserved in RNA later at −70 °C. The approximate region of the motor cortex from each brain was excised and minced by mechanical breakage in Precellys (3 cycles of 5000 rpm for 5 s each, followed by a cycle of 6000 rpm for 5 s). Similarly, thoracic spinal cord was excised and minced.

RNA from brain, spinal cord and from IPSCs derived cells was extracted with Qiazol (Qiagen) according to the manufacturer's instructions. RNA integrity was analyzed with 1.5% agarose gel and 2% hypochlorite [33]. In short, 1000 ng of RNA was reverse transcribed into cDNA using Superscript III reverse transcription kit (Invitrogen). Quantitative real-time PCR was performed using 20 ng of cDNA and 2X SYBR Green PCR Master Mix (Applied Biosystems) in a 7500 Fast Real-time PCR equipment (Applied Biosystems). For in vivo experiments, murine primers used were: GFAP, *CD11b, NF-kB, SOD1* and *CAT*. For in vitro experiments (with cDNA from IPSCs derived cells), human primers were: *SOD1, CAT, GSR* and *GPX*. All sequences are listed in supplementary material (Table S1). GAPDH was used as endogenous control and relative expression was calculated using Pfaffl's method [34] .

The presence of injected cells was verified through expression of human ACTB (β-actin; Hs01060665_g1) in cDNA obtained from brain and spinal cord used for in vivo gene expression analysis. For that purpose, the same qRT-PCR conditions were used but 250 ng of cDNA was used in each reaction.

Statistical Analysis

Results obtained with the macrophage phagocytosis assay and cytokines quantification were analyzed by non-parametric Mann Whitney test. For all analysis, the significance level was $p = 0.05$.

Gene expression was calculated according to sex and organs (brain or spinal cord). For each set of analysis, normalization was performed by both individuals from the vehicle group, separately. Expression data were initially evaluated by non-parametric Kruskal-Wallis tests. Existing differences were compared in pairs with Mann Whitney tests.

Survival of SOD1 mice was initially assessed by Kaplan-Meier estimators. Survival functions were compared with log-rank tests [35]. To assess the joint effect of sex and treatment on survival, Cox *proportional hazards* regression analysis were considered [36] and log-likelihood ratio tests [35], with 5% significance level were used to identify significant effects. The results based on the fitted model were expressed in terms of confidence intervals (95% confidence coefficient) for the relative risks.

The longitudinal data generated by physical performance tests applied during the course of the disease in all animals were analyzed by mixed linear models [37], according to the following steps:

a) Identification of the moment when animals become symptomatic (symptoms onset) and there is a change in the phenotype (onset of weight loss, for example) for the six groups defined by the combination of treatment (HBSS, MSCs, pericytes) and sex (male, female). For this step we used the algorithm proposed by Muggeo [38];
b) Comparison of the instant of symptoms onset among the six groups and identification of those common to two or more groups;
c) Use of the instant of symptoms onset (obtained in b) for fitting segmented regression models with the objective of estimating the *expected rate of variation in response* (weekly loss of weight for example) for each group (or set of groups);
d) Evaluation of the effect of treatment, sex and their possible interaction on the *expected rate of variation in response* after the symptoms onset.

Results

Cell Line Establishment and Characterization

As expected, MSCs population were positive only for CD13, CD29, CD44, CD73, CD90, CD105 and HLA ABC [39]and were able to differentiate into adipocytes, osteoblasts and chondroblasts. For pericytes however, the adipogenic potential was absent (supplementary Fig. S1). The integrity of subtelomeric regions was confirmed for MSCs and pericytes through MLPA analysis, before the starting of the experiments (supplementary Fig. S1).

Evaluation of Immunomodulatory Potential of MSCs and Pericytes

The amount of MSCs or pericytes after co-culture with SOD1 derived macrophages did not differ significantly from the quantity of MSCs or pericytes that were cultured alone (supplementary Fig. S2). In contrast, macrophages were capable to phagocytize the India Ink dye when it was added for 2 h in the culture medium (positive control). Additionally, no cellular formation resembling granulomas were observed for any co-culture, meaning that MSCs and pericytes are possibly able to enter the peritoneal cavity of SOD1 mice without being phagocytized by macrophages.

MSCs and pericytes were co-cultured with lymphocytes in order to assess the immunosuppressive potential of these cells. Results (supplementary Fig. S3) revealed a dose-dependent suppression of lymphocyte proliferation beginning at 5.10^4 MSCs or pericytes. These results show that MSCs and pericytes possess similar immunosuppressive potential, which is limited to the amount of cells present in the inflamed microenvironment.

Survival Analysis

Kaplan-Meier survival curves are presented in Fig. 1. In line with previous studies [24, 25, 40], male SOD1 had lower survival when compared to females, independently from the treatment (data not shown). Considering the three treatments (pericytes, MSCs and HBSS), regardless of the gender, no significant differences were observed between the survival curves. For females, treatment with MSCs or pericytes had no positive effect ($p = 0.76$) (Fig. 1a). However, for males, the survival curves were significantly different ($p = 0.04$) (Fig. 1b).

In order to assess the influence and magnitude of the differences observed in the initial analysis, a more detailed statistical analysis (based on Cox proportional hazards model) was performed considering sex, treatment and interaction between these two as prognostic factors. The survival of males did not differ significantly when treated with MSCs or HBSS ($p = 0.48$), while a significant increase was observed after treatment with pericytes ($p = 0.02$). A new model was fitted considering six groups formed by the combination of sex and treatment. The results indicated no differences in the survival curves for treated females ($p = 0.82$) and no differences for males treated with MSCs and HBSS ($p = 0.36$) but suggested a significant difference of the survival curves for males treated with pericytes and HBSS ($p = 0.01$), reproducing the findings

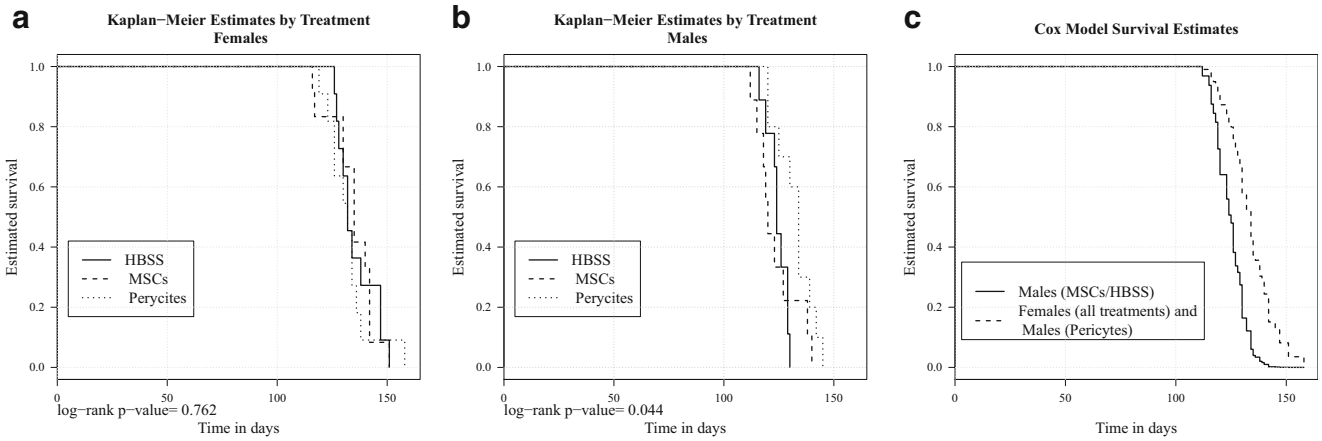

Fig. 1 **a-c** Kaplan-Meier estimates of survival and log-rank test ($n = 22$ mice per group, being 11 males and 11 females). Influence of treatment on female (**a**) and male (**b**) SOD1 mice and (**c**) final adjusted mathematical model of the two formed groups: (1) all females and males that received pericytes and (2) males treated with MSCs and HBSS

of the initial analysis. Additionally, the survival curve for the female group (combining the three treatments) was significantly higher compared to that of HBSS or MSCs treated males ($p < 0.01$ and $p = 0.01$, respectively) as expected according to the observed milder severity of the phenotype in females. Interestingly, males receiving pericytes showed survival curves similar to that of females ($p = 0.50$).

The final fitted model (Fig. 1c) considers two groups: one including females (regardless of treatment received) and males treated with pericytes and another group comprising males receiving HBSS or MSCs. On average, the group of males receiving MSCs or HBSS lived a total of 124 days compared to 133 days for the group of males treated with pericytes and all females. This represents a 7.3% increase in survival for males that received pericytes in comparison to the other males.

Analysis of Physical Performance Tests and Weight Evaluation

Although there are some statistical evidences of differences, the results observed with physical performance tests and weight evaluation are not consistent and lack a biological explanation (Fig. S4 and Table S2). Therefore, we concentrated our conclusions on the survival data.

Gene Expression Analysis and Identification of Human RNA by Real-Time PCR

Fully paralyzed animals were considered as end-stage and were sacrificed with ketamine overdose. Brain and spinal cord were extracted for gene expression analysis and human RNA identification (from injected cells).

Results obtained with gene expression analysis from in vivo experiments are shown in Fig. 2. We observed a significant decrease in expression of *CD11b* gene in female brains treated with MSCs or pericytes (no significant difference between these two groups), suggesting the existence of a less aggressive microenvironment. Additionally, genes encoding antioxidant enzymes (*SOD1* and *CAT*) were overexpressed in brain of males treated with pericytes. Overexpression of *SOD1* was also observed in brains of females that received either MSCs or pericytes, with no difference between these two groups.

In addition, real time PCR was used to search for traces of human RNA as an indication of the presence of injected cells in neuronal environment (data not shown). We could not identify the presence of human RNA in brain or spinal cord of SOD1 treated mice.

Expression of Oxidative Stress Related Genes in co-Culture System of Pericytes or MSCs with Human Motor Neurons and Other Neuronal Cells (MNNs)

iPSC derived motor neurons and other neuronal cells (MNNs) were validated through the evaluation of neuronal markers expression (Fig. S6). Co-culture with MSCs for 7 days significantly stimulated the expression of *CAT* in patient-derived MNNs. Both *SOD1* and *CAT* expression were increased after co-culture with pericytes (Fig. 2c).

Cytokine Quantification in a co-Culture System of Pericytes or MSCs with Human Motor Neurons and Other Neuronal Cells (MNNs)

Our results demonstrated that co-cultures of pericytes did not influence the amount of cytokines in the culture media of patient-derived MNNs (supplementary Fig. S5). On the other hand, co-culture of MSCs promoted a significant increase in the amount of diverse cytokines, being IL-6 and IL-8 the ones

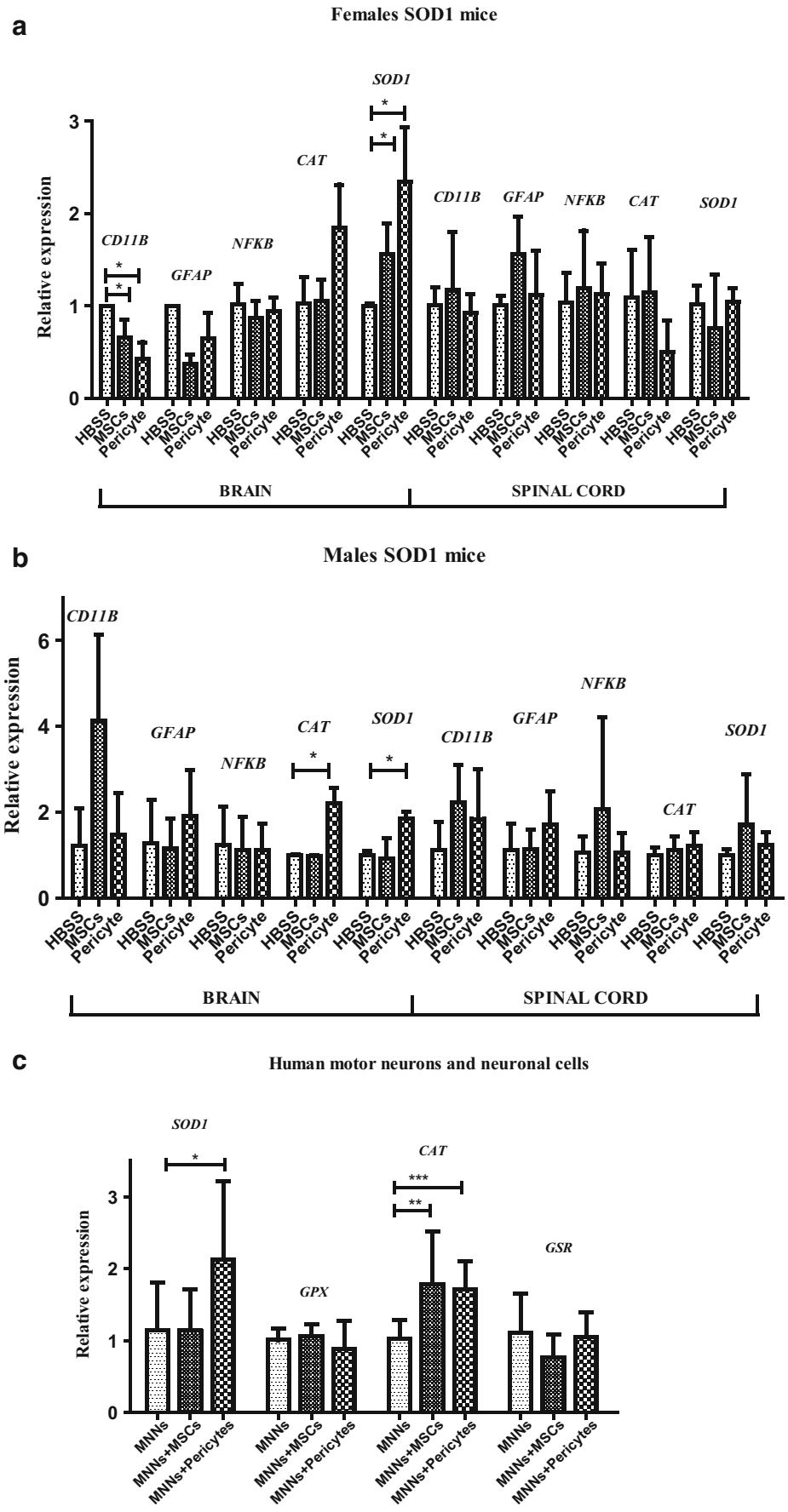

Fig. 2 Gene expression analysis. In vivo evaluations ($n = 4$ per group, being 2 males and 2 females) of brain (motor cortex enriched) and spinal cord of end-stage treated females (**a**) and males **b** SOD1 mice. **c** In vitro evaluation of patient derived motor neurons and other neuronal cells after co-culture with MSCs or pericytes or without co-culture. For neuronal differentiation, iPSCs from one ALS patient was used. The experiment was carried out in four replicas. $^{*}p < 0.05$ $^{**}p < 0.01$ $^{***}p < 0.001$

with the most significant increase (66 and 61.4 fold, respectively).

Discussion

Here we evaluated the therapeutic effect of human pericytes in SOD1 mice, the murine model for ALS. Since an ideal cell therapy protocol should be as little aggressive as possible, we first evaluated weekly intravenous delivery of cells in a small group of SOD1 mice (data not shown). However, in this pilot study, SOD1 mice frequently died as a result of the procedure, particularly in a later stage of the disease. Therefore we chose the intraperitoneal route, since it represents a minimally invasive method and was proven by others to be an efficient route of cell delivery for neurodegenerative disorders in mice [41].

When cells are transplanted trough the intraperitoneal route, besides reaching the brain, they are directed to other organs such as liver, and lungs [42]. Therefore, to compensate for the dispersion of cells throughout the animal body, we performed weekly injections until the animals natural death.

Pericytes were chosen for evaluation in SOD1 mice due to their well-established protective role against neuronal damage in neurodegenerative diseases [16]. The therapeutic effect of pericytes have been evaluated for other pathologies such as muscle [10] and bone disorders [43]. Recently, human pericytes, purified from skeletal muscle, improved heart indexes and engrafted within the host myocardium in a mouse model for ischemic heart disease, demonstrating that pericytes can improve tissue repair even if they are obtained from a different organ system and even from a different specie [11].

Although our pericyte lineage did not differentiate into adipocytes, this capacity is not mandatory for pericytes classification, as occurs for MSCs [39] but implications of this inability are not known for human pericytes. For mice however, subpopulations of pericytes that possess the ability to differentiate into adipocytes present different biological properties in comparison to subpopulations that lack this potential [44].

In order to analyze the effect of treatment with pericytes or MSCs, gene expression analysis by real-time PCR was performed in the final stage of the disease. Reduction in *CD11b* expression (related to the amount of activated microglia) and simultaneously increase in murine *SOD1* expression (encoding the antioxidant enzyme superoxide dismutase 1) was observed in brains of females treated with MSCs and pericytes, with no significant differences between these groups. This result suggests that, despite no significant effect on survival and clinical condition were observed in females, treatment with MSCs or pericytes promoted a less aggressive neuronal environment.

Interestingly, although MSCs arise from pericytes [13–15], in the present study, the effect of treatment with pericytes was more effective than treatment with MSCs. Males treated with pericytes have a survival rate similar to that of females, which is naturally higher than that of males [25].

Differences in severity according to gender has been reported for several autosomal disorders [45–47]. The influence of gender has been widely studied in ALS patients. The incidence and prevalence of ALS is higher in men than in women, and men are the majority amongst younger patients. The gender does not appears to have a clear effect on survival [26]. Several hypotheses have been proposed to explain this inherent difference such as hormonal influences and particularities between male and female nervous system, including different ability in repairing the damage of these tissues [26].

Numerous studies have reported differences in electrophysiological parameters, motor abilities and also in survival between male and female SOD1 mice carrying G93A mutation and with B6SJL genetic background, the same lineage used here [24, 25, 40]. Since females have a comparatively milder form of the disease, the therapeutic effect of these cells should be more pronounced in order to result in a significant clinical impact. Also, several reports demonstrated that the biological role of transplanted cells is highly dependent on the general condition that is found in the host microenvironment [48–51], which may also trigger different responses in males versus females.

Through the lymphocyte proliferation assay we observed that MSCs and pericytes possess a similar immunosuppressive potential, limited to the amount of cells present in the inflamed microenvironment. However, cytokine quantification in the medium of co-cultures of MSCs or pericytes with human IPSCs-derived MNNs demonstrated that MSCs treatment induces the secretion of diverse cytokines, being IL-6 and IL-8 the ones with the most significant increase in comparison to MNNs alone. IL-6 and IL-8 may possess dual functions according to the composition of the microenvironment but they are frequently linked to a pro-inflammatory profile [52, 53]. Priming MSCs with TLR3 stimulating agents prior to transplantation, in order to polarize cells towards an anti-inflammatory profile could help to improve the results observed here for this cell line [51].

Gene expression analysis showed a significant increase in *SOD1* and *CAT* expression in the brains of males treated with pericytes, suggesting that the inner antioxidant system was stimulated. Several reports demonstrated the involvement of reactive oxygen species in ALS pathogenesis [54–56]. Markers related to the oxidative stress damage of lipids, proteins or nucleic acid have already been detected in *post-mortem* tissue from ALS patients [55, 56] so that antioxidant enzymes or by-products of oxidative stress are considered as biomarkers for this disease [57].

Some antioxidants have already been evaluated for the treatment of ALS with some positive effects in disease progression of SOD1 mice [58, 59]. Treatment with the PET-imaging agent CuATSM and Copper-Chaperone-for-SOD (CCS) allowed delivery of copper into the CNS and

consequently helped maturation of SOD enzyme by inserting copper in its molecular structure. Cu-Zn-SOD protein amount was increased and animals survival was extended [60].

In order to validate the results obtained in SOD1 mice we used an "in vitro" experimental system. Human iPSCs derived motor neurons and other neuronal cells (MNNs) from one ALS patient carrying a *FUS* mutation were co-cultured with MSCs or pericytes trough transwell inserts, allowing the passage of soluble factors and mimicking the hypothesized paracrine effect of these cells. The presence of MSCs in co-culture induced the expression of *CAT* in MNNs while pericytes induced the expression of *SOD1* and *CAT*, in accordance with the results observed in male SOD1 mice.

IPSCs derived cells have been used for disease modeling [32] and recently also for drug discovery [61, 62]. Our results demonstrated that this co-culture system could also represent a useful cellular model for complementary studies of great importance to evaluate the potential effect of cell therapy since neither in vivo (with SOD1 mice) or in vitro experiments (with iPSCs derived MNNs) are totally informative about what might happen within the patient's host tissue. Gain and/or loss of function experiments or even in vitro assays with human motor neurons carrying different mutations will give further support to the present findings in future studies.

Our results suggest that the beneficial effect of injected pericytes in SOD1 males was probably due to a paracrine effect through soluble factors, since we were not able to trace injected cells in animals brains or spinal cords. However, we believe that it is very important to determine the fate of transplanted cells in future experiments. For that purpose, pericytes obtained from NG2-DsRed mice, that naturally express a red fluorescent protein, could be followed in SOD1 mice after transplantation.

We propose that pericytes are able to exert their therapeutic effect in males SOD1 mice by stimulating the host antioxidant system. Soluble factors secreted by MSCs were found to stimulate the expression and activity of antioxidant enzymes in fibroblasts from patients with Friedreich's ataxia, protecting against oxidative damage [63, 64].

Recently, a small molecule namely Ro-31-8425 promoted targeting of systemically infused MSCs to sites of inflammation, and boosted their therapeutic impact [65]. In addition, antioxidant treatment of MSCs prior to transplantation was found to enhance their anti-stress ability and therapeutic efficacy in an acute liver failure model [66]. The possibility to increase pericytes homing and survival within the neural microenvironment could also improve their therapeutic effect. Following this line of thought we also believe that it is important to evaluate other routes of cell delivery, including intrathecal transplantations, in order to enhance delivery to the central nervous system.

To our knowledge, this is the first study evaluating the therapeutic effect of pericytes in pre-symptomatic SOD1 mice, a period in which there are cellular abnormalities but no clinical manifestation [23]. Evaluating the effect of this treatment in symptomatic mice will be of great interest. However, the present experimental design could be translated to patients in the early phases of ALS, such as those affected with the familial form.

In short, our results suggest that pericytes are able to exert their therapeutic effect in males SOD1 mice by stimulating the host antioxidant system through the release of soluble factors, an effect apparently not reported before for SOD1 mice. Although we have no explanation for why this effect is restricted to males, extending their survival to the same observed in SOD1 females is a positive outcome. Additionally, the results obtained here with mice carrying a mutation in SOD1 gene and with human iPSCs-derived-MNNs from an ALS patient carrying a mutation in *FUS,* suggest that the beneficial effect of pericytes could hopefully be translated to patients with ALS harboring diverse mutations.

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Compliance with Ethical Standards

Disclosure of Interest The authors indicate no potential conflicts of interest.

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