

Bone marrow-derived mesenchymal stem cells versus adipose-derived mesenchymal stem cells for peripheral nerve regeneration

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Abstract

Studies have confirmed that bone marrow-derived mesenchymal stem cells (MSCs) can be used for treatment of several nervous system diseases. However, isolation of bone marrow-derived MSCs (BMSCs) is an invasive and painful process and the yield is very low. Therefore, there is a need to search for other alternative stem cell sources. Adipose-derived MSCs (ADSCs) have phenotypic and gene expression profiles similar to those of BMSCs. The production of ADSCs is greater than that of BMSCs, and ADSCs proliferate faster than BMSCs. To compare the effects of venous grafts containing BMSCs or ADSCs on sciatic nerve injury, in this study, rats were randomly divided into four groups: sham (only sciatic nerve exposed), Matrigel (MG; sciatic nerve injury + intravenous transplantation of MG vehicle), ADSCs (sciatic nerve injury + intravenous MG containing ADSCs), and BMSCs (sciatic nerve injury + intravenous MG containing BMSCs) groups. Sciatic functional index was calculated to evaluate the function of injured sciatic nerve. Morphologic characteristics of nerves distal to the lesion were observed by toluidine blue staining. Spinal motor neurons labeled with Fluoro-Gold were quantitatively assessed. Compared with sham-operated rats, sciatic functional index was lower, the density of small-diameter fibers was significantly increased, and the number of motor neurons significantly decreased in rats with sciatic nerve injury. Neither ADSCs nor BMSCs significantly improved the sciatic nerve function of rats with sciatic nerve injury, increased fiber density, fiber diameters, axonal diameters, myelin sheath thickness, and G ratios (axonal diameter/fiber diameter ratios) in the sciatic nerve distal to the lesion site. There was no significant difference in the number of spinal motor neurons among ADSCs, BMSCs and MG groups. These results suggest that neither BMSCs nor ADSCs provide satisfactory results for peripheral nerve repair when using MG as the conductor for engraftment.

Key Words: nerve regeneration; mesenchymal stem cells; adipose-derived mesenchymal stem cells; sciatic nerve; Matrigel; sciatic functional index; neural regeneration

Introduction

Despite the recent progress towards repairing peripheral nerve trauma, complete functional recovery does not occur due to the failure of damaged peripheral nerves to regenerate. Therapies aimed at nerve regeneration are particularly challenging in cases with significant loss of nerve segments, for which nerve grafts are needed. However, the use of autologous nerve grafts to connect damaged peripheral nerves is limited by the availability of donor nerves and the damage that can occur at the donor sites during the extraction for transplantation (Millesi, 1984, 1993; Sabongi et al., 2015).

To facilitate peripheral nerve gap repair, biological or synthetic tubes can be used as conductors. Biological tubes include muscle tissue or vessels, whereas synthetic tubes can be constructed from non- or biodegradable materials, such as silicone, alginate gel sponges, collagen sponges, and polyglycolic acid. These tubes are designed to create a microenvironment enabling neurotrophic factors to stimulate neural regeneration and reconnection of nerve extremities *via* chemotaxis (neurotropism) (Politis et al., 1982; Longo et al., 1983). Biological tubes from vein segments have been widely used for axonal

regeneration (Lolley et al., 1995; Colonna et al., 1996; Ferrari et al., 1999), as veins can be easily isolated with minimal injury to the patient, in contrast to the damage caused when obtaining nerves for autologous nerve grafts, and the procedures involved are less expensive than other methods. Thus, veins represent an ideal source of autogenous material (Sabongi et al., 2015).

Bone marrow-derived mesenchymal stem cells (BMSCs) have been identified as an alternative for many therapies for cell or tissue damage, including that of the nervous system, with numerous ongoing clinical trials (Donnenberg and Ulrich, 2013; Nery et al., 2013; Souza et al., 2014). Mesenchymal stem cells (MSCs) likely contribute to neurogenesis by inducing the secretion of different neurotrophic factors either directly from local precursors or indirectly from nearby activated astrocytes (Uccelli et al., 2011). However, the procedures for isolating BMSCs are invasive and painful with a low yield of obtained stem cells (Zuk et al., 2001). Therefore, other sources of MSCs have been explored for possible use in transplantation therapy. Adipose tissue-derived MSCs (ADSCs), which have phenotypic and gene expression profiles similar to those of BMSCs (De Ugarte et al., 2003; Strem et al., 2005; Nery et al., 2013), can be collected from

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subcutaneous fat tissue using conventional liposuction. Moreover, the yield of ADSCs from adipose tissue exceeds that of BMSCs (Gimble et al., 2007), and ADSCs proliferate at a higher rate than BMSCs (Yoshimura et al., 2007; Liao et al., 2010). ADSCs were used in peripheral nerve grafts showing better results than nerve grafts without the ADSC cells (Liu et al., 2011). Thus, we compared venous grafts containing BMSCs or ADSCs to regenerate lesioned sciatic nerves in rats to determine if ADSCs represent a feasible alternative for nerve autografts.

Materials and Methods

Animals

In this study, 40 male and 20 female 8-week-old isogenic spontaneously hypertensive rats, weighing about 250 g, were provided by the Experimental Model Center of the Federal University of São Paulo (CEDEME-UNIFESP). The animals were provided *ad libitum* access to a standard diet and water and maintained on a 12-hour light/dark schedule. Experiments were designed with the 3R (replacement, refinement, and reduction of animals in research) concept in mind. Animal protocols were approved (No. 1880/10) by the institutional ethical committee and were conducted in accordance with guidelines of the Brazilian College of Animal Experimentation and the National Institutes of Health for the care and use of laboratory animals.

Rats were randomly divided into four groups. In the BMSCs group, rats received BMSCs-containing MG after sciatic nerve injury ($n = 10$). In the ADSCs group, rats received ADSCs-containing MG after sciatic nerve injury ($n = 10$). In the Matrigel (MG) group, rats received MG vehicle only after sciatic nerve injury ($n = 10$). In the sham group, rats underwent a sham operation only as a reference for functional normality following nerve exposure ($n = 10$).

BMSCs extraction

BMSCs were obtained using a previously described protocol (Fernandes et al., 2008), with some modifications. Briefly, marrow from the femoral and tibial bones of isogenic female rats ($n = 10$) was flushed in a laminar flow using a 3-mL syringe containing Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA). A 70- μ m nylon mesh filter was used to isolate BMSCs, which were then centrifuged for 5 minutes at $200 \times g$ at 4°C . The floating fractions were discarded, and each of the pellets was re-suspended in 25 mL MSC medium (DMEM containing 10% fetal bovine serum [FBS] and 1% penicillin-streptomycin [Gibco, New York, NY, USA]). The cells were incubated at 37°C with 5% CO_2 for 1–2 weeks, during which the medium was replaced every 2–3 days (Zhang et al., 2010). When the cultures achieved 80% confluence, the cells were treated with trypsin-EDTA and collected by centrifugation as above. Cell viability was verified by Trypan blue (Gibco) staining, and 2×10^6 viable cells were mixed with 20 μL of MG (Sigma-Aldrich, St. Louis, MO, USA) for engraftment (Zhang et al., 2010).

ADSCs extraction

The inguinal areas of female rats ($n = 10$) were dissected to obtain fat tissue (~ 10 g), which was rinsed with saline and digested in filtered (0.20- μ m syringe filter) 0.075% collagenase at 37°C for 1 hour. Low-glucose DMEM (Thermo Fisher Scientific) containing 10% FBS and 1% penicillin-streptomycin was added to inhibit the enzymatic activity, and the cells were pelleted by centrifugation at $600 \times g$ for 10 minutes. The pellets were re-suspended and incubated for 3 minutes in 160 mM ammonium chloride at

room temperature and then filtered (70- μ m nylon mesh). The cells were again pelleted and re-suspended in DMEM and cultured in 75- cm^2 flasks (Nunc, Roskilde, Denmark) at 37°C under 5% CO_2 , with medium changes every three days. When the cultures achieved 80% confluence, the adherent cells were collected using 0.25% trypsin-EDTA, washed with phosphate-buffered saline, and stained with Trypan blue to determine cell viability (Tholpady et al., 2003). For engraftment, 2×10^6 viable cells were mixed with 20 μL of MG.

Surgical procedures

Animals were anesthetized with injections (0.4 mL/100 g body weight, i.p.) comprising 4.25 g chloral hydrate, 2.25 g magnesium sulfate, 42.8 mL propylene glycol, 11.5 mL ethyl alcohol, and 45.7 mL of distilled water. Surgical procedures have been described elsewhere (Fernandes et al., 2008). Briefly, the sciatic nerves were dissected from the subfascial muscles between the vastus lateralis and the biceps femoris and severed. Eight-millimeter segments were resected, leaving a ~ 3 -mm stump proximal to the nerve ramifications. To obtain vein segments for engraftments, 12-mm segments of posterior external jugular veins were carefully resected using a latero-lateral transcervical approach, and stored in a saline solution as described previously (Sabongi et al., 2014). Each of the jugular veins received MG only or MG containing BMSCs or ADSCs to repair the 8-mm sciatic nerve gaps. The veins were sutured to the epineuria with 10-0 monofilament nylon. For the sham surgery, the right sciatic nerves were only exposed (not severed) for 10 minutes, and the muscles were sutured with 4-0 monofilament nylon. The parameters observed in the sham group should be close to normal and can be used in comparative analyses among groups that underwent invasive procedures (Fernandes et al., 2008; Kizilay et al., 2017).

Sciatic functional index (SFI) assessment

SFI was obtained for each group (sham, MG, ADSCs and BMSCs). The rats were trained to walk through a 43 cm \times 8.7 cm tunnel towards a shelter (De Medinaceli et al., 1982). The hind paws of the animals were then painted with ink to obtain footprints as they walked the track after surgery at 30-day intervals for 90 days. All footprints were scanned using an HP PSC 1610 scanner (Hewlett-Packard, Palo Alto, CA, USA) and measured (in mm) to calculate Bain's parameters (Bain et al., 1989). SFI was calculated as $[-38.3(\text{PL}_E - \text{PL}_N)/\text{PL}_N] + [109.5(\text{TS}_E - \text{TS}_N)/\text{TS}_N] + [13.3(\text{IT}_E - \text{IT}_N/\text{IT}_N)] - 8.8$, where PL_E and PL_N represent the print lengths, TS_E and TS_N represent the total toe spreads, and IT_E and IT_N represent the intermediate toe spreads of the experimental and normal limbs, respectively.

Morphologic and morphometric analyses

One hundred and twenty days after surgery, five rats from each group were randomly selected for morphometric and morphologic analyses. Sciatic nerve segments (5 mm in length) distal to the grafts were removed for morphologic and morphometric studies as previously described (Fernandes et al., 2008; Sabongi et al., 2014). Briefly, nerve segments fixed in 2.5% glutaraldehyde were incubated in 2% osmium tetroxide with 0.2 M sodium cacodylate (1:1) for 12 hours and dehydrated with ethanol and propylene oxide before embedding in plastic resin (Epon 812; Electron Microscopy Sciences, Hatfield, PA, USA) as described previously (Sabongi et al., 2014). Semithin (0.5 μm) sections were then cut on a Reichert Jung ultramicrotome (Leica-Microsystem, Wetzlar, Germany) and stained with toluidine blue.

Briefly, the internal borders of perineuria from transverse

sections were imaged under a 10× objective on a Zeiss Imager MI AX10 microscope (Carl Zeiss AG, Oberkochen, Germany) using a camera (JVC TK 1270) with a 1× optovar and processed with the Picture Frame software AxioVision LE (Carl Zeiss, Oberkochen, Germany). We measured the nerve areas and minimum diameters along the inner borders of the perineuria using ImageJ 1.45 software (National Institutes of Health, Bethesda, MD, USA) as described previously (Sabongi et al., 2014). The numbers of fibers were quantified in two of four quadrants from each field. The analyzed area fractions and densities of myelinated nerve fibers were recorded. The fiber density (number of fibers/mm²), fiber diameters, axonal diameters, myelin sheath thicknesses, and G ratios (the axonal diameter/fiber diameter ratio) were determined from binarized images.

Fluoro-Gold (FG) staining

Motor neurons in the anterior horns of the spinal cords were retrogradely labeled with FG, as previously described (Fernandes et al., 2008). Briefly, 120 days after surgery, incisions were made to expose the injured sciatic nerves to a 3% FG (Fluoro-chrome, Denver, Colorado, USA) solution for 90 minutes and then sutured. Forty-eight hours later, the animals were transcardially perfused and segments of the spinal cords from L₃ to S₁ were cryoprotected and frozen for sectioning (40-μm slices). The numbers of FG-positive motor neurons strongly positive for FG in the anterior horns of the spinal cords under fluorescence microscopy Zeiss-Axiolab (Carl Zeiss, Oberkochen, Germany) were determined in accordance with the correction criteria described by Abercrombie (1946) which is an estimation of nuclear population from microtome sections.

Statistical analysis

Statistical analyses for significant differences of experimental data were performed using analyses of variance (ANOVAs) with Bonferroni *post-hoc* tests through Statistica software (StatSoft, Palo Alto, CA, USA). Differences were considered significant when $P < 0.05$.

Results

Sciatic nerve function

Sham-operated rats ($n = 9$) exhibited similar SFIs before and after the procedures ($P > 0.05$). Rats in the BMSCs and ADSCs groups ($n = 10$ each) and the MG group ($n = 6$) exhibited slight improvement over the duration of the study (Table 1), with SFIs that were significantly lower than those of rats in the sham group ($P < 0.001$). Moreover, SFI score in the BMSCs group was higher than that in the ADSCs group on day 30 ($P < 0.01$) and in the MG group on day 60 ($P < 0.001$).

Morphometric and morphologic analyses results of the nerve distal to lesion

The nerve fascicles of rats in the sham group showed normal endoneural spaces and G ratios and no myelinated fiber loss or signs of Wallerian degeneration, myelin sheath thinning (suggestive of demyelization), or axonal sprouting (Figure 1A). By contrast, nerve fascicles from rats in both the BMSCs and ADSCs groups exhibited a loss of large-diameter myelinated fibers. Many small-diameter fibers were observed, suggesting axonal sprouting that occurred either in intra- and extrafascicular spaces or surrounding the fascicular borders (Figure 1B–D). In the BMSCs group, Wallerian degeneration was detected in a few fibers, with no alterations of the neural and epineural spaces, indicative of axonal regeneration in chronic axonal lesions (Figure 1B). Although not as pronounced, the nerve fascicles from animals in the MG group also had few large-caliber myelinated fibers and groupings of small-caliber fibers, with a few fibers exhibiting signs of Wallerian degeneration (Figure 1D).

Data from the morphometric analyses are shown in Table 2. The mean axonal diameter and myelin sheath thickness in the sham group ($n = 5$) were $6.24 \pm 0.2 \mu\text{m}$ and $2.63 \pm 0.2 \mu\text{m}$, respectively. By contrast, the BMSCs and ADSCs groups ($n = 5$ each) had narrower fibers, with mean axonal diameters of $3.25 \pm 0.8 \mu\text{m}$ and $3.19 \pm 0.8 \mu\text{m}$, respectively. The mean axonal diameter in the MG group was $2.69 \pm 0.1 \mu\text{m}$. The diameters of the fibers and axons in the BMSCs, ADSCs, and MG groups were significantly smaller than those in the sham group (all $P < 0.001$). The myelin sheaths were also significantly thinner than those in the sham group (all $P < 0.001$). However, G ratios and fiber densities were similar among the groups.

Number of motor neurons

A significantly higher number of FG-labeled motor neurons in the rat anterior horns was observed in the sham group ($n = 10$) than in the BMSCs, ADSCs, and MG groups ($n = 10$ each) (Table 3). Interestingly, the mean numbers of motor neurons were similar between the BMSCs and ADSCs groups ($P > 0.05$). The MG group also achieved similar results to experimental groups ($P > 0.05$).

Discussion

Our results demonstrate that venous grafts with either BMSCs or ADSCs did not induce recovery as assessed by SFI values, with poor outcomes relative to the sham controls. Nevertheless, a slight but significant functional improvement was observed on day 60 in rats receiving BMSCs compared with in rats receiving only MG.

A comparison of our results with those from other peripheral nerve repair studies was difficult due to the large variability in

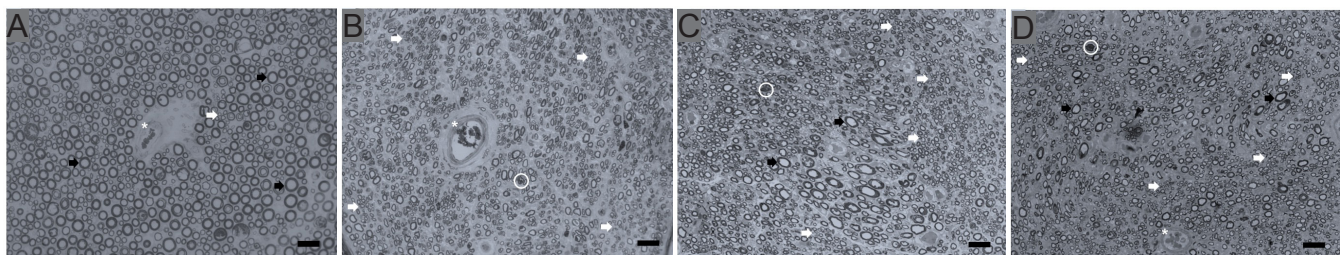


Figure 1 Sciatic nerve segments from sham controls (A) and from animals receiving grafts containing BMSCs (B), ADSCs (C), or MG only (D). Sections stained with toluidine blue reveal large-caliber (black arrows) and small-caliber (white arrows) myelinated fibers. Blood vessels are marked with asterisks and sites of Wallerian degeneration are indicated by white circles. Calibration bar is 20 μm at a magnification of 400× using a Zeiss Imager MI AX10 microscope. BMSCs: Bone marrow-derived mesenchymal stem cells; ADSCs: adipose-derived stem cells; MG: Matrigel.

Table 1 Sciatic functional index value in each group

Group	n	SFI at postoperative days			
		0	30	60	90
Sham	9	-11.78±11.21	-7.18±11.99	-11.50±11.25	-10.48±6.39
MG	6	-10.63±10.20	-76.31±17.32*	-87.87±7.57*	-93.18±10.02*
ADSCs	10	-6.81±8.94	-79.86±12.59*	-71.18±10.68*	-91.34±10.66*
BMSCs	10	-12.96±7.73	-59.60±17.20†	-61.21±13.86‡	-91.14±11.07*

Data are expressed as the mean ± SD. **P* < 0.001, vs. sham group; †*P* < 0.01, vs. ADSCs group; ‡*P* < 0.001, vs. MG group using analysis of variance with Bonferroni *post-hoc* test. MG: Matrigel; ADSCs: adipose-derived mesenchymal stem cells; BMSCs: bone marrow-derived mesenchymal stem cells. Higher sciatic function index values represent better functional outcome.

Table 2 Morphologic and morphometric analyses of nerve segments in each group

Group	n	Analyzed area (µm ²)	Fiber density (number/mm ²)	Fiber diameter (µm)	Axon diameter (µm)	No. of myelinated fibers	Thickness of myelin sheath (µm)	G ratio
Sham	5							
Mean±SD		152.80± 51.10	17.69±2.31	11.51±0.37	6.24±0.25	635±280	2.63±0.20	0.55±0.01
Range		128.00–244.00	15.00–20.81	11.09–11.90	5.98–6.61	480–1,170	2.44–2.96	0.54–0.56
MG	5							
Mean±SD		59.20±33.40	48.92±10.58	4.90±0.16*	2.69±0.11	748±505	1.11±0.04*	0.55±0.01
Range		20.00–96.00	39.00–66.22	4.78–5.15	2.58–2.82	210–1,523	1.07–1.17	0.54–0.56
ADSCs	5							
Mean±SD		52.00±31.37	47.95±17.70	5.45±1.23*	3.19±0.84	658±217	1.13±0.20*	0.57±0.02
Range		20.00–104.00	27.58–69.70	3.84–6.83	2.13–4.28	279–717	0.86–1.36	0.55–0.61
BMSCs	5							
Mean±SD		64.80±38.41	35.67±13.93	5.85±1.18*	3.25±0.87	622±270	1.30±0.17	0.54±0.05
Range		16.00–104.00	25.00–59.50	3.78–6.56	1.78–3.92	106–736	1.00–1.42	0.47–0.59

**P* < 0.001, vs. sham group *via* analysis of variance with Bonferroni *post hoc* test. MG: Matrigel; ADSC: adipose-derived mesenchymal stem cells; BMSCs: bone marrow-derived mesenchymal stem cells.

Table 3 Number of Fluoro-Gold-labeled motor neurons in the anterior horn of rat spinal cord in each group

Group	n	Number of motor neurons
Sham	9	678.71±16.11
MG	6	542.50±19.88*
ADSCs	10	548.90±24.44*
BMSCs	10	508.72±45.72*

Data are expressed as the mean ± SD. **P* < 0.001, vs. sham group *via* analysis of variance with Bonferroni *post-hoc* test. MG: Matrigel; ADSCs: adipose-derived mesenchymal stem cells; BMSCs: bone marrow-derived mesenchymal stem cells.

experimental parameters. The sizes of the nerve gaps and the types of stem cells used vary among different preparations. However, SFI on day 30 in our BMSCs group was -59.60, whereas a value of -49.94 was described by Zheng and Cui (2012), who used a chitosan conduit filled with BMSCs to repair 8-mm gaps. In another study using polycaprolactone nerve conduits filled with BMSCs to restore 3-mm gaps, an SFI score of approximately -90.00 was reported (Frattini et al., 2012). A study using ADSCs injected in allograft nerves with gaps of 1.5 cm reported a mean SFI of -80.51 on day 90 (Liu et al., 2011); we found an SFI of -91.34 for the corresponding group in our study.

In our previous study using adult BMSCs in agar as the vehicle for the engraftment of sciatic nerve gaps (8 mm), we determined low SFI on days 30 and 60 following surgery, with values of -61.3 and -68.3, respectively (Fernandes et al., 2008). By

contrast, rats receiving BMSC transplants in the present work had higher SFIs than those in the ADSCs group on days 30 and 60 and those in the MG group on day 90 following surgery. The SFI score decreased over time, reflecting the altered gait of rats following denervation of the hind limb.

We found that the fiber densities increased in all experimental groups, with the highest overall density in the MG group, though the differences were not statistically significant. These increases and the nerve injury-induced axonal sprouting were attributed to increases in the numbers of small-diameter (~7-µm) fibers. These findings are consistent with previous studies showing that the number of fibers increased up to 3 months after nerve injury, reached a plateau at 6–9 months, and returned to baseline values after 1 year (Dellon and Mackinnon, 1989). We also measured axonal diameter to fiber diameter ratios (G ratios), which are an index of nerve impulse conduction. A normal G ratio, reflecting efficient nerve conduction, is approximately 0.6, and we observed a ratio of 0.55 in the sham group. Values approaching 0 indicate axonal atrophy, whereas those closer to 1 reflect demyelination (Friede and Samorajski, 1967). The G ratios observed in the BMSCs (0.54), ADSCs (0.57), and MG (0.55) groups indicate that the different treatments applied did not alter nerve conduction.

The numbers of motor neurons, as determined by FG staining did not differ between the BMSCs and ADSCs groups. Under both experimental conditions, the functional results were below what was expected. One point to consider is that ADSCs can differentiate into adipocytes, which could have contributed to the lack of improvement in relation to the use of MSCs. Another point to consider is that the regenerative effect of the

MSCs is dependent on the density used (Jiang et al., 2017), and perhaps we used an insufficient amount to obtain the expected result. The lower than expected recovery is similar to what was observed in our previous study using BMSCs and agar rather than MG as the vehicle (Fernandes et al., 2008). MG is a matrix scaffold of the extracellular matrix, which includes laminin, collagen IV, and enactin, and it is widely used to maintain the self-renewal and pluripotency of stem cells, keeping them in an undifferentiated state (Widgerow et al., 2014). Despite the high quality of MG for stem cell culture, the expected results have not been achieved for ADSCs and BMSCs in this study.

In conclusion, our study showed that neither BMSCs nor ADSCs provide satisfactory results for peripheral nerve repair when using MG as the conductor for engraftment. Further studies are necessary to investigate the factors involved with MSCs engraftment for nerve repair in animal models.

Author contributions: MJSF, VML, HU and MF made substantial contributions to conception and design of the study. MF, SGV and RGS performed surgical procedures and collected the data. SGV, HU, and AAN performed preparation of stem cells, morphometric and morphologic analysis, and motoneuron counting. MJSF, SGV, MF, RGS, JBG analyzed and interpreted the data, contributed to preparation of the manuscript, and revised it for publication. All authors approved the version of this manuscript.

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Research ethics: Experiments were designed with the 3R (replacement, refinement, and reduction of animals in research) concept in mind. Animal protocols were approved (no. 1880/10) by the institutional ethical committee and were conducted in accordance with guidelines of the Brazilian College of Animal Experimentation and the National Institutes of Health for the care and use of laboratory animals.

Data sharing statement: Datasets analyzed during the current study are available from the corresponding author on reasonable request.

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