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Intravenous human dental pulp-derived mesenchymal stem cell therapy for ischemic stroke in rats: an analysis of functional and ischemic brain areas outcomes

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Abstract: Cellular therapies have been implicated in treating and rehabilitating ischemic stroke (IS), involving the basic experimental and preclinical areas. Using mesenchymal stem cells (MSC) derived from human dental pulp has shown promising results in animal models, but still with mechanisms and consequences that are not entirely clear. The study aims to evaluate the effects of intravenous MSC on rats with IS regarding neurological function and histological areas of ischemia. Thirty-two male Wistar rats underwent temporary occlusion of the middle cerebral artery (TOMCA) for 60 minutes and were divided into two groups of 16 animals each. One group received dental pulp MSC intravenously, and another received saline 2 hours after TOMCA. The animals were then evaluated using the neurological functionality scales for 15 days, and at the end of the experiment period, the histological areas of cerebral ischemia were analysed. All animals presented ischemic areas and neurological deficits compatible with IS. There was partial recovery of the functionality scores over the evaluation period, and all animals presented focal cerebral ischemia measured by histological analysis; however, there was no statistical difference between the groups. The TOMCA model was effective in reproducing IS. Although we found no difference between treatment groups, our results were useful in showing the pattern of neurological recovery presented by animals treated with dental pulp MSC and the need to extend the evaluation time for a longer period and use more sensitive functional tests. The results add valuable data for improving research with dental pulp MSC in the murine model of IS.

Keywords: Ischemic stroke; Cell therapy; Mesenchymal stem cells; Temporary middle cerebral artery occlusion; Rehabilitation

1.0 INTRODUCTION

Ischemic cerebrovascular disease is a significant public health problem, the second leading cause of morbidity and mortality worldwide. Recent data show that the global burden of deaths and sequelae on survivors has been increasing worldwide, especially in developing countries (GBD 2016 Neurology Collaborators, 2019). A series of health system problems means that only a small portion of the population suffering from an ischemic stroke (IS) has timely access to treatment in the acute phase, which must be carried out within a limited period. Even in specialised centres, only a portion of less than 15% of cases receive treatment in the acute phase and less than 3% in general hospitals, including the Brazilian reality (Katzan et al., 2000; Nakiri et al., 2017).

In this context, most patients remain without effective treatment to assist their recovery and rehabilitation in the late stages of the ischemic event, significantly impacting their quality of life and the economy. In this sense, seeking new therapies that enable functional improvement is essential. These therapies should preferably be able to be carried out with a longer time interval between the initial event and its application, making them more viable and benefiting a more significant number of people. Among these therapies, several research groups have studied using mesenchymal stem cells (MSC) in stroke (Kocsis & Honmou, 2012).

The experimental model most used to study focal cerebral ischemia has been the permanent or temporary obstruction of the middle cerebral artery (TOMCA) in rats (Kusaka et al., 2004; Ma et al., 2020). Temporary artery occlusion mimics the process that commonly occurs in ischemia of large vessels, with a period of occlusion followed by reperfusion from that arterial territory, which mimics the reperfusion process that occurs in humans, either by chemical, mechanical or endogenous thrombolysis (Calloni et al., 2010).

MSCs are present in the dental pulp, an excellent source of which teeth extracted for clinical/ dental indications (which would usually be discarded) could be used to harvest them (Inoue et al., 2013). These cells have a high capacity for self-regeneration, immunomodulation and proliferation and can differentiate into different tissues, including nervous tissue. MSCs are multipotent cells with great immunosuppressive and regulatory capacity and important therapeutic perspectives for treating neurological diseases. They stimulate tissue recovery and regeneration by secreting numerous anti-inflammatories, angiogenic, neurotrophic, immunomodulatory and antifibrotic factors (Chopp & Li, 2002).

Initially, it was believed that MSC therapy for neurological diseases could replace damaged cells. Still, current studies suggest several alternative mechanisms MSCs trigger, such as releasing trophic factors that promote neuroprotection, neovascularisation, remyelination and axonal sprouting, which are also useful for treating neurological diseases (Sasaki et al., 2016). These factors improve the functioning of neurons, promote greater survival, decrease inflammation, and increase angiogenesis. Therapies with MSCs aim to protect neurons that are still viable in each injury or disease and not only replace damaged cells and tissues (Leong et al., 2012; Mendez-Otero et al., 2009).

Although treatment with MSC several hours after the start of the stroke can reduce the size of the infarction area and improve functional recovery in rodent cerebral ischemia models (Chen et al., 2001; Horita et al., 2006; Vu et al., 2014), the mechanism by which MSC promotes these benefits has yet to be further studied. Thus, some issues still need to be better detailed concerning the use of MSC in the IS, such as the best MSC extraction site, the ideal number of cells, the cell inoculation site, the perfect time for the cells to exert their effects, possible side effects/ biosafety of the procedure and the degree of recovery developed by the animals (Chopp & Li, 2002).

Most preclinical studies of stem cells in stroke used bone marrow-derived MSC, with fewer studies evaluating the effects of dental pulp MSC. Therefore, a greater degree of detail about functional effects, effect size and their correlation with cerebral ischemic areas is needed for the development of dental pulp MSC research in stroke (Nito et al., 2022). Beneficial effects found by research with a viable source of MSC, such as those derived from dental pulp and with an intravenous route of administration, would be of great value and bring us closer to a possible clinical translation.

Studies using MSCs derived from the pulp of human teeth in research with rats demonstrate positive results in IS models (Shi et al., 2020; Song et al., 2017; Yalvac et al., 2009). MSCs derived from dental pulp showed positive results in stroke models (Shi et al., 2020; Wang et al., 2022), and these results were not inferior to those MSCs derived from bone marrow (Song et al., 2017). Therefore, investigating the effects

exerted by the injection of human dental pulp MSC in rats with focal cerebral ischemia, both from the point of view of altering neurological function and the area of cerebral ischemia, was the primary justification and objective of the present work.

More specifically, the objectives of this study were to analyse comparatively, between the groups, the neurological functionality scores achieved in the open field test (by the number of displacements/rearings) and in the modified neurological severity scale (mNSS) in 1, 7 and 15 days after the stroke. The study also aimed to comparatively analyse the areas of cerebral ischemia in coronal histological sections of the brain 15 days after the ischemia between the treated and the control groups.

2.0 MATERIALS AND METHODS

2.1 Animal model and experimental groups

The Ethics Committee on the Use of Animals in the Federal University of Rio Grande do Sul (UFRGS) approved this project (Protocol number: 32595). Thirty-two male Wistar rats (*Rattus norvegicus*) were used for the experiment, each weighing approximately 270-320g and aged between 8 and 10 weeks at the beginning of the experiments. The animals were kept in plexiglass boxes measuring 27 x 26 x 31 cm covered with wood shavings (4 per box), received feed and water *ad libitum*, and remained in a 12-hour light/ dark cycle. All animals underwent one week of adaptation to the laboratory environment before the experiments.

The animals underwent a surgical procedure temporarily obstructing the left middle cerebral artery (TOMCA). After 1 hour of obstruction, normal circulation was resumed by removing the obstructive factor, allowing brain reperfusion. After 24 h of reperfusion on D1 after stroke, the animals were divided into two groups of 16 animals, and each received an amount of $2x10^6$ MSCs in a volume of 0.5 mL or the same volume of 0.3% saline (control group) through the vein of the animal's tail. After that, the neurological function tests were conducted on animals in standardised times of 24 hours, 7 days and 15 days after reperfusion. After 15 days, the animals were anaesthetised and euthanised with 5% isoflurane, and their brains were analysed to quantify the ischemic area in stained histological sections (**Figure 1**). The researcher responsible for functional tests was blind to the type of treatment received.

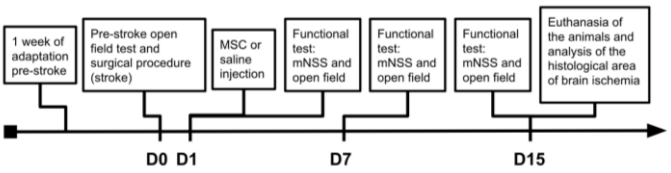

Figure 1. Experimental design and stages in which animals were studied. D0: the day of the stroke; D1, D7, D15: the first, seventh, and fifteenth day after the stroke. Mesenchymal stem cells (MSC) were injected on D1 (24 hours after the stroke). The control group received saline in the same time interval. Functional tests such as the modified Neurological Severity Scale (mNSS) and the open field test were performed at the indicated times.

2.2 Surgical procedure

All surgical procedures were performed by the same researcher (Greggianin G. F.). **Figure 2** describes the procedure steps obtained by the surgical microscope recording.

2.2.1 First anaesthetic time

Anaesthetic induction was performed with 5% isoflurane in a closed system, with a machine developed for laboratory anaesthesia and external oxygen support, followed by 1.5% maintenance in 30% O_2 and 70% N_2O. The anterior cervical region trichotomy was performed by using a trichotome and blade. The rat was positioned in a bed heated to 35-37°C, fixed with adhesives and ventilated with an adapted helmet. Antisepsis was performed using alcoholic chlorhexidine and local anaesthesia with 0.2mL of 0.5% Lidocaine.

Paramedian incision and dissection of subcutaneous planes under microscopy (Zeiss S88 surgical microscope with magnification of up to 8x) with microsurgical instruments allowed the identification of mandibular gland, sternomastoid muscle laterally, sternum muscle medially and digastric muscle superiorly, which together form a triangular space whose interior is the anatomical reference to find the carotid bifurcation.

Careful dissection of the carotid sheath was performed to expose the common carotid artery (CCA), the proximal part of the internal (ICA), external carotid artery (ICA) and to separate these structures from the venous plexus and vagus nerve (**Figure 2a**). Local hemostasis was achieved using cotton buds, cotton and bipolar cautery if necessary. A 5-0 prolene suture was positioned around the CCA with a single knot without restricting vessel flow. Distal and proximal CCA

were clipped with a minimum distance of 0.5 cm between the clips. A delicate arteriotomy in CCA was performed with a hypodermic 26G needle or microscissors. Then, a 4-0 nylon filament was introduced in CCA with a thick silicone capped tip (marketed by Doccol Corp., Redlands, USA) (**Figure 2b**).

Delicate closing of the knot and opening of the distal clip allowed the cranial progression of the 4-0 nylon filament. The white tip of the filament could be seen through transparency entering the ICA. It proceeded until resistance was found or a small part of the thread remained outside the vessel (approximately 3 cm from the entrance to the CCA), at which point the thick tip of the filament would obstruct the outlet of the middle cerebral artery (MCA). Hemostasis was verified by keeping the proximal clip and the skin closed. Next, the animal was hydrated with 10 mL/kg of 0.3% NaCl (injected intraperitoneally), followed by removing the animal from the anaesthesia machine.

After one hour counted from the moment of filament progression (ischemia time), the animal went to the second stage. Before the second anaesthetic period, the researcher performed a clinical test to verify ischemia by lifting the animal by the tail and making it walk freely on the plane, observing signs of right hemiparesis.

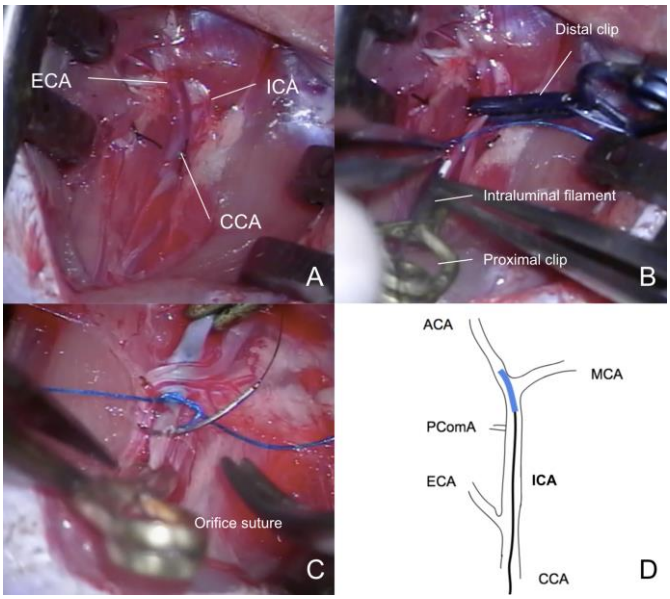

Figure 2. (a) Microsurgical view after left cervicotomy, with exposure of arterial vessels. **(b)** Introduction of the white-tipped filament through the arteriotomy orifice. **(c)** Suture of the orifice after removal of the intraluminal filament. **(d)** Schematic illustration of filament placement occluding the flow in the MCA. External carotid artery (ECA), Internal carotid artery (ICA), Common carotid artery (CCA), Posterior communicating artery (PComA), Anterior cerebral artery (ACA), Middle cerebral artery (MCA).

2.2.2 Second anaesthetic time

Anesthesia was performed following the first procedure, removing the subcutaneous suture and identifying previous microsurgical aspects. The endovascular filament was pulled out until direct view through the transparency of the white-coloured silicone-capped tip. The distal clip was repositioned, and the knot loosened. The arteriotomy orifice in the CCA was sutured directly with 9-0 nylon thread (**Figure 2c**). The distal clip was then carefully opened, followed by the opening of the proximal clip to verify hemostasis. In the end, we closed the skin and applied postoperative analgesia with 50 micrograms of tramadol subcutaneously on the animal's back.

2.3 Isolation and transplantation protocol for mesenchymal stem cells (MSCs)

MSCs were isolated, cultured and characterised previously according to protocols standardised in the Hematology and Stem Cell laboratory at UFRGS (Bernardi et al., 2011). The established cell line was used for later transplantation in animals subjected to ischemia/ reperfusion of the left MCA. Pulp cells from human primary teeth were trypsinised and resuspended with Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with 10% Bovine Fetal Serum (FBS), plated in culture bottles and kept in a 37°C humid atmosphere at a 5% CO_2 for 72 hours with culture medium changes every 3-4 days. When the culture reached a confluence of 90%, subcultures were performed. The cells were characterised and frozen according to international standard protocols (Bernardi et al., 2011; Dominici et al., 2006).

MSCs were characterised immunophenotypically in the fifth and tenth passage through the presence of MSC marker markers, such as CD29 (BD, 561795), CD44 (BD, 553135), CD73 (BD, 550257), CD90 (BD, 554897) and CD105 (BD, 559135), as well as confirmation of the absence of hematopoietic stem cell markers, such as CD34 (BD, 559369), CD45 (BD, 559135) and HLA-DR (BD, 562007) using the flow cytometry technique. A total number of 106 cells was incubated with the conjugated antibodies cited above for 30 min at 4°C. Acquisitions were made using BD FACSAria equipment with two lasers (Becton Dickinson, Franklin Lakes, NJ, USA). Isotypic controls for FITC and PE were used. The gating strategy was performed as previously described (Bernardi et al., 2011). Living cells were evaluated for

monoclonal antibodies against the antigens listed previously.

The differentiation potential necessary to confirm the population's identity obtained was tested from the fifth passage using standardised osteogenic, adipogenic and chondrogenic differentiation assays in the stem cell laboratory as described previously (Bernardi et al., 2011).

After 24h of ischemia-reperfusion, animals in the treatment group received $2x10^6$ MSCs intravenously resuspended in 0.5mL of DMEM-low medium supplemented with fetal bovine serum (Xu et al., 2014) and animals in the control group received 0.5mL 0.3% NaCl. For this purpose, the animals were briefly anaesthetised (as described in the surgical procedure section), and the lateral caudate vein was identified and catheterised with a sterile 24G abocath.

2.4 Open field testing

To perform this test, each animal was placed in the left quadrant of an open field arena (40 cm x 50 cm x 60 cm dark box). White lines were drawn on the ground to divide the bottom surface of the field into 12 equal quadrants. This test was filmed using a digital camera (Samsung ES80), and each animal's number of crosses over the quadrants (with all four paws) and rearings were monitored. This test was a parameter for animals' locomotor and exploratory activity. The three-minute behavioural test was carried out at a consistent time in the morning for the duration of the study with standard light as previously described (Sosa et al., 2015).

2.5 Modified neurological severity score (mNSS)

The modified Neurological Severity Score (mNSS) combines neurological tests assessing sensory, motor and reflex responses (Ruan & Yao, 2020). It consists of a series of functional assessments with a score for each item. The higher the sum of the scores, the worse the animal's neurological status (the score ranged from 0 to 18, where 1–6 indicated mild injury, 7–12 represented moderate injury, and 13–18 indicated severe injury).

2.6 Analysis of ischemic area

For the analysis of the ischemic area, a previously described protocol was used (Wang-Fischer, 2009). Initially, the animals were anaesthetised (isoflurane 3% in 30% O_2 and 70% N_2O) and then beheaded with a guillotine. The animals' brains were dissected, removed from the skull, and kept in a refrigerator at -18 °C for 30 min. After these procedures, coronal cuts of 1 mm were made with the aid of a support/ matrix, disregarding the regions of the cerebellum and olfactory bulb. The tissue slices obtained were incubated for 30 minutes in a solution of 2,3,5-triphenyl tetrazolium chloride (TTC) 2% at 37 °C and fixed in 4% paraformaldehyde overnight (Arrick et al., 2012; Taniguchi & Andreasson, 2008).

The stained sections were digitised with a high-resolution scanner and analysed using free image analysis software (ImageJ software [National Institutes of Health (NIH), Bethesda, MD, USA]). TTC stains tissues with vitality, so the devitalised area (infarction area) looks paler and normal areas become a deeper red. The ImageJ software with the colour saturation tool was used to help delimit the most intensely coloured (red) and the least (white) areas. The areas were manually delimited by a researcher who was blind to the group of animals. The ischemic area (IA) in each slice was defined as a percentage (%) of the contralateral hemispheric area (CHA) which is free of ischemia or oedema. The ischemic area (IA) was defined as the contralateral hemispheric area (CHA) minus the ipsilateral normal area (INA), and this result was divided by CHA and multiplied by 100, that is: IA = [(CHA - INA)/ CHA] x 100.

Due to an expected loss of some slices (tissue fragmentation occurred in the process), not all specimens had the same number of slices available for analysis. Therefore, the choice of slices to be analysed took into account the three largest areas in absolute terms, as they correspond to the most representative areas of ischemia in that brain. The IA of each animal was calculated by averaging the IA of these three most representative slices.

The results were expressed as means ± standard deviation (SD). Statistical analysis and graphs were assessed or generated using JASP version 0.13.1. The student's t-test was used to compare paired normal samples with a significance level of $p<0.05$. The Mann-Whitney test was used to compare unpaired non-normal samples, and the Wilcoxon test for paired non-normal samples. Both tests with a significance level were defined as $p<0.05$.

3.0 RESULTS

Three of the 32 animals died, one in the intraoperative period, probably attributed to anaesthesia complications and two on the first postoperative day of unknown aetiology. One of the animals was resistant to the effects of the anaesthetic gas and was excluded due to the impossibility of surgery. The group that received the treatment with MSC had 13 animals, and the control two cell surface markers simultaneously using group

had 15 animals. Only one animal did not show evidence of a motor deficit in the immediate postoperative period. However, this animal showed a neurological deficit in subsequent tests and presented histological brain ischemia. Therefore, it was not excluded from the analysis. In the sample, 27/28 (96.4%) animals presented clinical evidence of stroke in the immediate postoperative period (assessing gait and lifting the animal by the tail, presenting contralateral hemiparesis to the operated side).

3.1 Modified neurological severity score (mNSS)

The mean/standard deviation (SD) of the control group mNSS was 5.4 (SD = 2.87) on D1, 2.8 (SD = 1.01) on D7 and 2.2 (SD = 0.94) on D15. The mean / SD of the treatment group mNSS was 6.61 (SD = 3.22) on D1, 3.92 (SD = 2.46) on D7 and 2.15 (SD = 0.98) on D15. The mean mNSS values and the variability progressively reduced over the 15 days for both groups (**Figure 3**). The mNSS scores on D1, with no effect of any intervention after surgery, were similar between the groups ($p = 0.301$ in the student's t-test), showing a similarity of the initial neurological status between groups.

The data was organised for comparative purposes, referring to the differences between the mNSS values at different evaluation times for each animal. The greatest difference was observed between the D1 and D15 mNSS in both groups (mean reduction of 4.46 points for the treatment group and 3.2 points for the control group). The groups had no statistical difference ($p > 0.05$ in the Mann-Whitney test).

3.2 Number of crossings/rearings in the open field test

The number of crossings /rearings in both groups in the preoperative period (D0) was, as expected, statistically higher than those in the other periods evaluated, with a mean of 88.06 (SD = 20.1) displacements for the control group and 84.40 (SD = 14.15) for the treatment group ($p < 0.05$). The number of crossings/ rearings decreases mainly on the first day after the stroke, with partial recovery over the 15 days of evaluation for both groups (**Figure 4**).

For the control group, there was a significant difference between the number of preoperative displacements (D0) and all other times and between D1 (mean = 21.42 / SD = 25.86) and D7 (mean = 54.57 / SD = 32.79) (Wilcoxon p-value < 0.05) but not between D7 and D15 (mean = 38.84 / SD = 29.64). For the treatment group, there was a significant difference ($p < 0.05$ in the student's t-test) between the number of preoperative (D0) crossings/ rearings and all other times and between D1 (mean = 10.3 / SD = 8.25) and D7 (M = 30.92 / SD = 26.12) and D1 and D15 (M = 43.15 / SD = 24.57), but not between D7 and D15.

There was no statistically significant difference between the number of crossings/ rearings between control and treated animals in any evaluation times ($p > 0.05$ in the Mann-Whitney test).

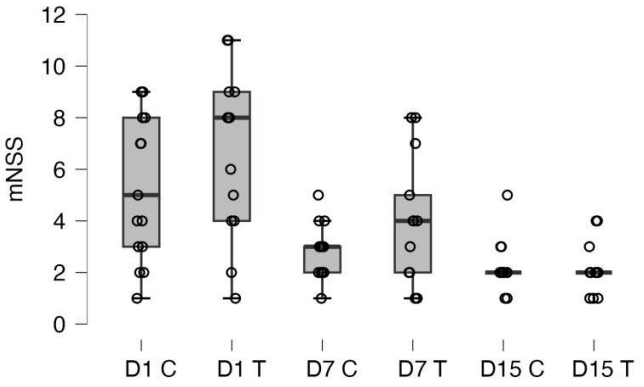

Figure 3. Modified Neurological Severity Score (mNSS) values for each animal (dots) in control (C) and treatment (T) groups at the first (D1), seventh (D7) and fifteenth (D15) day post-stroke. The boxplot represents each group at each evaluation time. The mean mNSS values and variability progressively reduced over the 15 days for both groups.

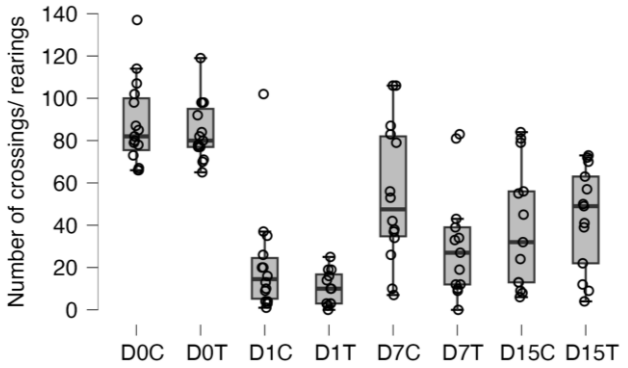

Figure 4. Number of crossings/rearings performed by the animals in the open field test during 3 min. On the x-axis, the control (C) and treatment (T) groups were identified for each evaluation day, with D0 immediately before, D1, D7, and D15 the first, seventh and fifteenth day after the stroke. Both the control and treatment groups had a higher number of crossings/rearings immediately before stroke (D0), followed by partial recovery over the subsequent 15 days. There was no statistical difference between groups ($p > 0.05$ in the Mann-Whitney test).

3.3 Analysis of the histological area of brain ischemia

Analysis was performed regarding the relative ischemia area as a percentage of the corresponding hemisphere area. There were some sample losses, with nine brains analysed in the control group and 13 in the treatment group, as some sections suffered tissue disintegration, not allowing their analysis. All 22 brains were examined histologically and stained with TTC, showing some area of ischemia, and these were always located in the MCA territory ipsilateral to the operated side (temporarily occluded). Although the mean percentage of ischemic area in the control group was 47.03% (SD = 11.31) and that of the treated group was 36.86% (SD = 15.57), there was no significant difference between the groups ($p > 0.05$ in Student's t-test) as shown in Figure 5A. Figure 5B shows a scanned coronal slice of the brain stained with TTC on the left and, on the right, the same slice analysed using the Image J software, highlighting the parts stained by TTC in red.

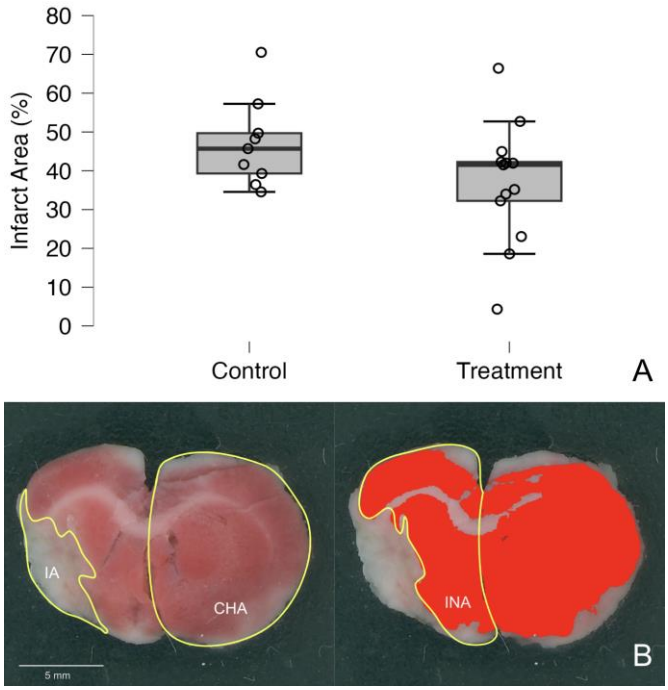

Figure 5. The infarcted areas of each animal in the percentage of the normal corresponding area (CHA) are represented as dots in Figure 5A and the boxplot of these data from each group. Figure 5B shows a coronal slice of the brain stained with TTC on the left and, on the right, the same slice analysed using the ImageJ software, highlighting the parts stained by TTC in red. A researcher blinded to the groups manually delimited the contralateral hemispheric areas (CHA) and the ipsilateral normal area (INA). The ischemic area (IA) was defined by the formula IA = [(CHA - INA)/ CHA] x 100.

4.0 DISCUSSION

Animal disease models are essential for research and allow testing in an ethical and controlled manner of different therapies. In the stroke field, there are three types of stroke models: global ischemia (involving the four cerebral irrigation vessels), focal ischemia (such as the TOMCA) occlusion model and lacunar (multifocal) stroke models. Among the focal ischemia models, TOMCA in rats is one of the most important and used in focal stroke research (Hossmann, 1998). As detailed in the methodology, our model presents a slight difference from the most used standard model as previously described (Calloni et al., 2010).

In the current model, a small orifice in the CCA was made to introduce the filament, and after the procedure, this orifice was sutured, taking care not to occlude or stenosis of the vessel. The standard model introduced the filament through the ECA towards the ICA. In the current study, 96.4% of the animals presented clinical evidence of stroke in the immediate postoperative period. In addition, all 22 brains analysed histologically and stained with TTC showed some area of ischemia, and this was consistently located in the MCA territory ipsilateral to the operated side, showing that the model was effective in determining focal ischemia in the MCA territory with an occlusion time of approximately 60 minutes and was 100% effective in determining the focal area of ischemia in the analysed sample of 22 animals. The group noticed that it became easier to introduce the filament with a direct path towards the animal's ICA. However, suturing the orifice in the CCA requires greater microsurgical training to avoid strictures and haemorrhages. In the sample, there was no death from intraoperative haemorrhage, and the researchers could perform the technique properly on all animals, suggesting this is an effective and safe model.

The mNSS is a widely used and validated tool to assess the global neurological function of rodents (Chen et al., 2001). It was found that there was a pattern of progressive recovery over the evaluation period. The group treated with MSC had an average recovery of 4.46 points, and the control group had 3.2 points on the scale when comparing the scores on D1 with D15. This data is compatible with the recovery pattern observed by other authors in similar works in which the scale was used for follow-up after TOMCA (El Amki et al., 2017; Nito et al., 2022). Although the mean recovery of points in the treatment group was greater

than that of the control group, no statistical difference was found between the groups. Perhaps a more extended follow-up period could make this difference more evident, as seen in a study in which the observation period was 28 days (Song et al., 2017). MSCs exert their therapeutic effects in different ways (Wu et al., 2020), and the neuro-repair mechanisms provided by MSCs may need more time to become clinically evident. This hypothesis was supported by the observation that the improvement of mNSS continues to occur even after 15 days of stroke in a study that evaluated the use of MSCs after TOMCA (Song et al., 2017).

Compared to the preoperative period, the number of crossings/ rearings in the open field test showed a sharp drop pattern on the first day after the stroke (D0). This pattern was expected due to the hemiparesis presented by the animals, with similar motor impairment between groups. During the evaluation period, there was a partial recovery in the number of displacements, but they were not equal to the preoperative period. The statistical difference was observed between the number of displacements from D1 compared to D7 and D15 but not between D7 and D15 for the two groups. This shows a potential for early motor recovery (in the first week) impacting the number of animal movements, which did not last in the second week. Perhaps this is due to a recovery plateau regarding crossings/ rearings in this test. There were no differences between groups at any time during the open field test. The number of animal movements is influenced not only by the animal's motor capacity but also by the animal's motivation to explore the environment, presence of pain/discomfort, vision and possible distractions such as noises and smells. Despite these limitations, the test was easy to apply and an indirect indicator of the rodent's neurological and general health status, which is characterised by exploring the environment in which it is found.

The animals did not undergo rehabilitation training after the stroke. In a study conducted by El Amki and colleagues, it was observed that rehabilitation training in a task-directed at the affected forelimb of the animals favoured their recovery in this task and even in other tasks with increased mNSS along 28 days (El Amki et al., 2017). Interestingly, in that study, the two groups (with and without rehabilitation) had no differences in the areas of ischemia assessed by brain resonance (T2 sequence) in 28 days. One possible explanation for this is that neurorehabilitation promotes cellular mechanisms of regeneration that are not necessarily evident as a reduction in the appreciable ischemic area in MRI scans. The lack of a rehabilitation program after a stroke may prevent or reduce the effectiveness of MSCs in fully exercising their effects on functional improvement.

There were some losses in the areas of ischemia, with nine brains analysed in the control group and 13 in the treatment group, with some slices suffering tissue disintegration, not allowing their analysis. With fewer animals and slices, the analysis may not have reached a sufficient sample number to show a significant difference. However, the percentage of ischemic areas in the treatment group was lower than in the control group. Using the staining technique with TTC is fast, feasible in most laboratories, inexpensive and effective as a method of detecting and quantifying the ischemic brain area of rodents (Bederson et al., 1986; Joshi et al., 2004). However, despite having a good correlation, it is not yet 100% equal to the ischemic area analysed by electron microscopy (Benedek et al., 2006; Park et al., 1988). Cases in which the colouration is very faint make analysis difficult with the naked eye, and automated analysis tools by colour saturation, such as the one that we used (ImageJ software), were applicable to help the researcher delimit the infarcted areas. (Goldlust et al., 1996).

As previously noted, clinical improvement does not necessarily correlate with areas of infarction. It is also noteworthy that the injection of MSC occurred 24 hours after the stroke, and by this moment, the central ischemic damage was already established. Previous studies showed reduced ischemia with MSCs even after the injection 24 hours after the stroke (Song et al., 2017). Possible neuroprotective and anti-inflammatory mechanisms of MSCs can reduce secondary damage due to oedema and revitalise or prevent the definitive ischemia of penumbra areas even after several days of the initial injury (Li et al., 2021). In these studies, it is emphasised that the histological analysis took place with an interval of 28 days, allowing a more extended period for neurogenic or neuroprotective mechanisms to act, and perhaps this has been a limiting factor for our results.

Previous studies have indicated beneficial effects in vitro and in vivo in the recovery of rodents using dental pulp-derived MSCs in a TOMCA model by intracerebral injection of cells (Sugiyama et al., 2011) and by intravenous injection of MSC (Song et al., 2017). The intravenous injection is a more straightforward method to apply for possible future clinical tests, so it was

chosen over intracerebral or intra-arterial injections. The mechanisms of action, the potential for functional recovery, the potential for reducing the area of ischemia and the safety of intravenous use of dental pulp-derived MSCs are still open questions and whose research and investigation are precious for advancing cellular therapies in treating and rehabilitating stroke.

More than 700 neuroprotective drugs are reported and investigated in animal stroke models (Macleod et al., 2004), but with limited applicability in humans to date. This large number of drugs and therapeutic agents investigated, often with promising results in animal models but without appreciable effects in humans, demonstrates possible limitations and discrepancies that animal models have in relation to stroke in humans (Macleod et al., 2004). Some of the factors pointed out to explain these differences and limitations are the different ages (in humans, stroke occurs predominantly in the elderly and experimental studies use rats at relatively younger ages) and the absence of important comorbidities in animals such as obesity, hypertension, diabetes and cardiovascular disease (Kaya et al., 2017).

Despite limitations, basic animal research has contributed enormously to our knowledge of stroke. In a disease with such an important morbidity and mortality burden in our population, research for new therapies is necessary and improving animal models is extremely valuable for the scientific community. The present work has generated results of great value and reflections that will help improve new stroke therapy experiments using dental pulp MSCs.

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