Bone Marrow Mononuclear Stem Cell Transplant in Acute and Chronic Arterial Insufficiency in Rabbits

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Abstract

Introduction: There is a high incidence of Peripheral Obstructive Arterial Disease (POAD) in patients with atherosclerosis. In more complex cases for which surgical revascularization is not possible, the only option involves clinical treatment that in the majority of cases evolves to amputation of the limb. Transplant of mononuclear stem cells from bone marrow has presented favorable results in chronic obstructions.

Objective: To perform a functional analysis of the effect of bone marrow mononuclear stem cell transplant on acute arterial occlusion immediately and 48 hours after occlusion, comparing between groups and with controls.

Materials and methods: Twenty New Zealand rabbits were anesthetized with ketamine and xylazine (50 mg/kg) and underwent occlusion of the right iliac artery. Those animals that presented absence of arterial flow after ligation were included in the study. These animals were then randomized and divided into four groups: Group 1 (n=5) control of acute ischemia group—-injection of saline solution, Group 2 (n=5) control of chronic ischemia—-injection of saline solution 48hrs after occlusion. Group 3 (n=5) transplant of stem cells in the chronic ischemia group, 48hrs after occlusion. The animals were evaluated by the Tarlov’s movement scale, degree of tissue ischemia, and degree of modified ischemia on the seventh, fourteenth and thirtieth day after arterial occlusion. This evaluation was performed in a blind and randomized fashion by two different observers. The animals underwent another vascular Doppler exam on the thirtieth day after arterial occlusion.

Results: All animals were considered homogeneous in the pre-transplant period. No statistical differences were identified between groups G1 and G3 (p=0.109) with respect to Tarlov scale. Regarding the intergroup analysis, a clinical improvement was observed in Group 4 when compared to Groups 1, 2, and 3, p=0.003, p=0.0025, and p=0.055 respectively, on the thirtieth day after occlusion. No significant difference was observed for the degree of ischemia and modified ischemia parameters after transplant.

Conclusion: Clinical improvement in the chronic ischemia group receiving cell transplant of mononuclear stem cells was observed in comparison to the control group and in relationship to the acute ischemia group, suggesting a functional improvement in the affected limb.

Keywords: Arterial occlusion; Stem cells; Lower limb; Ischemia; Transplant

Introduction

Systemic cardiovascular disease can peripherally manifest and can thus become obstructive Peripheral Arterial Occlusive Disease (PAOD). Patients with PAOD are subject to acute ischemia in cases for which there is, for example, atherosclerotic plaque thrombosis or chronic ischemia, characterized by insidious occlusion of the vascular bed [1].

The most feared complication involving PAOD is the amputation of a lower limb. In the United States chronic ischemia is present in 12% of the population, of these patients, 50-70% remain stable; however, without treatment the risk of amputation can reach 1% per year [2].

Indication for treatment of PAOD requires a detailed and thorough evaluation, since atherosclerotic disease is systemic and thus involves a multidisciplinary staff. Rarely will these patients present, for example, an occlusion of the femoral artery or aorta without presenting coronary compromise; therefore a cardiology evaluation is primordial [1].

PAOD in conjunction with an arterial embolism or acute thrombosis imposes great difficulty in the treatment of this pathology since, in this type of situation; revascularization is more difficult and frequently even impossible. Therefore, the majority of patients remains untreated and runs a high risk of amputation [3].

Bone marrow stem cell transplant is a new option in treatment of patients with lower limb ischemia. Some studies have suggested a clinical improvement with the use of mononuclear stem cells from bone marrow. The results from bone marrow transplant in chronic ischemia of lower limbs suggest a functional improvement; however, in acute ischemia of the lower limbs, the results are not yet well known [4].

Accordingly, the objective of this study is to perform clinical analyses (motor and visual) of the effects of bone marrow mononuclear
stem cell transplant on acute arterial occlusion immediately and 48 hours after occlusion, comparing the groups to each other to control groups.

Materials and Methods

This project was presented to the Committee of ethics in research of Pontificia Universidade Catolica do Parana (PUCPR) and approved under protocol number 478 in September 2009. Twenty New Zealand (Oryctolagus cuniculus) male rabbits from the Biology Laboratory of PUCPR, weighing between 2000-3000 g and with ages of 24-28 weeks were included in the study.

Experimental model

The animals were anesthetized with ketamine and xylazine (50 mg/kg) followed by hair removal of the right lower limb for ligation of the iliac artery and the region of the posterior superior iliac crest in order to perform a bone marrow puncture and isolate the mononuclear stem cells.

The animals underwent a pre-operative Doppler evaluation of the lower limb for quantification of the arterial flow.

After blood flow evaluation, an incision was made in the inguinal region with a ligation of the right common iliac artery in order to induce ischemia in the rabbits’ lower limbs. Subsequently, a post-operative Doppler of the right lower limb was performed to quantify blood flow.

The inclusion criteria for the study required that the animals present no blood flow in the right lower limb after right iliac artery ligation.

After inclusion the animals were randomized and divided into four groups:

- **Group 1**: N=5. Control for acute ischemia–saline injection.
- **Group 2**: N=5. Control for chronic ischemia–saline injection 48 hrs after occlusion.
- **Group 3**: N=5. Stem cell transplant for acute ischemia.
- **Group 4**: N=5. Stem cell transplant for chronic ischemia, 48hrs after occlusion.

Subsequently, a surgical procedure in the lower limbs for the ischemia group was performed involving the transplant of bone marrow mononuclear cells and injection of saline for the control group. Cells were transplanted in the intramuscular layer of the entire right lower limb.

After forty-eight hours, the two chronic ischemia groups underwent the same surgical procedure. Transplant of bone marrow mononuclear cells in the study group and a saline injection in the control group was performed. As in the acute ischemia group, cells were transplanted in the intramuscular layer along the entire right lower limb. All animals returned to the animal laboratory and were followed for 30 days.

Motor evaluation and degree of ischemia

The clinical evaluation was based on the Tarlov scale, tissue ischemia degree, and modified ischemia. The Tarlov scale evaluates only the animals’ motor response and the other two evaluations compliment this score by limb examination [5] (Table 1).

This was a blind evaluation. Initially, the examiner evaluated the animals by observing the surgical wound and clinical signs of ischemia such as paleness and gangrene. After the initial exam, the gait was observed for 1 minute and 30 seconds, during such time any difficulty in mobilizing the hind paw or supporting weight was identified. All data was reported and filed.

These evaluations were performed on the seventh, fourteenth and thirtieth day after surgery.

After the thirtieth day, the animals were sacrificed with a lethal dose of pentobarbital.

Doppler vascular analysis

Vascular evaluations were performed using an Agilent, Sons 5500. The analyses were always done in a blind fashion by the same person.

Animals underwent anesthesia and hair removal of the right lower limb. Subsequently, they were placed in a dorsal decubitus position, using gel for interface and a linear transducer at a frequency of 8-13 MHz.

According to the inclusion criteria for the experiment, only those animals that did not present distal flow on the Doppler were selected. Prior to sacrifice, on the thirtieth day another Doppler test was done to evaluate the therapeutic response.

Preparation and isolation of cells, harvesting of bone marrow cells

Animals were anesthetized, placed in a lateral decubitus position and skin antisepsis was performed. Bone marrow was removed by multiple punctures in both posterior iliac crests with heparinized syringes of 5ml (Liquemine 5000 U/ml). The material was sent to the Experimental Laboratory of Cell Culture for isolation of mononuclear cells. One ml of heparin was used for each 100 ml of Dulbecco's Modified Eagle Medium (DMEM) culture medium.

Bone marrow suspension was diluted in an essential DMEM and placed slowly under a Ficoll-Hypaque density gradient (density=1.077 g/mL) in accordance with Boyum (1968). The material was centrifuged
at 1400 rpm for 40 minutes. The mononuclear cell ring in the interface was then placed in conic tubes containing 20 mL of DMEM. Cells were washed twice at 1500 rpm for 10 minutes and re-suspended with DMEM. Cell count was done using the Neubauer camera, cellular viability was verified using the vital dye Trypan Blue as well flow cytometric analysis.

**Flow cytometric analysis**

Flow cytometric analysis (FACS Calibur; Becton Dickinson, USA) was performed to validate the bone marrow origin of the stem cells. Immunophenotyping of CD34 and CD45 were performed with a commercially available kit using a single-platform method according to the International Society of Hematotherapy and Graft Engineering (ISHAGE) guidelines.

These kits consist of an anti-CD45 FITC (anti-CD45, Monoclonal-IgG1-3H1362-Santa Cruz Biotechnology, FITC, USA) and anti-CD34 PE (anti-CD34, polyclonal-IgG–671371-ABIN, PE, USA) and a respective isotype-matched control. These conjugated MoAbs are already provided in defined combinations ready to use for CD34 and for CD45 was needed to conjugate with secondary antibody.

All flow cytometric analysis was performed in duplicate, and the mean was calculated from the results.

**Histopathological staining**

After euthanasia, the anterior tibialis of the rabbits were removed and then were proceeded with standard preservation techniques for storage of biological tissue sample involve formalin-fixation and paraffin embedding. Serial transversal sections of 8 μ were stained with H&E and by immunohistochemistry with factor VIII (anti-factor VIII antibody, Dako, Copenhagen, Denmark) for histopathological assessments.

**Statistical analysis**

For comparison of the groups with respect to evaluation scores, the nonparametric Kruskal-Wallis test was utilized. The Friedman non-parametric test was used for the comparison of evaluation times between groups. Values of p<0.05 indicated statistical significance. Data was analyzed using the computer program Statistica v.8.0.

**Results**

**Flow cytometric analysis**

The cells were CD45+ and CD34− as immunophenotypical characteristics of mononuclear stem cells (Figure 1). The mononuclears cells obtained were 7.6 × 10⁶ ± 0.5 (Table 2).

**Analyses with vascular Doppler**

An analysis in B-Mode was performed for which smooth arterial walls were observed with no signs of atheroma in any of the animals. Doppler flowmetry was then performed. In the pre-operative flow analysis using a linear transducer, monophasic flow characteristics of the animals were observed, as well as a wave with a monophasic aspect in the distal femoral artery with little variation in amplitude prior to ligation of the right iliac artery.

After right iliac artery ligation, a new Doppler study was done to certify the absence of flow including any collateral vessels that could sustain flow in the animals’ lower limbs. All animals presented absence of flow in the right iliac artery.

In the Doppler performed on the thirtieth day absence of flow was identified; however, refilling was presented in some small collateral vessels.

**Analysis of variable movement by the Tarlov scale**

The following table shows the data for each animal included in the study (Table 3).

**Intragroup analysis**

In the period between the seventh and thirtieth days after tissue lesion there was no statistical difference identified in group G1 with p=0.109 and in group G3 with p=0.109. For groups G2 and G4 it was not possible to perform this analysis due to several tied scores identified for this period.

**Intergroup analysis**

In the period of seven days after lesion, groups G1 and G2 presented

![Figure 1: Mononuclear stem cells flow cytometric analysis from rabbit.](image)

<table>
<thead>
<tr>
<th>Group</th>
<th>Rabbit</th>
<th>7 days</th>
<th>14 days</th>
<th>30 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>1</td>
<td>5</td>
<td>5</td>
<td>4</td>
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<td></td>
<td>2</td>
<td>6</td>
<td>5</td>
<td>3</td>
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<td>5</td>
<td>6</td>
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<td>5</td>
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<tr>
<td>Median (min-max)</td>
<td>5 (3 - 6)</td>
<td>5 (4 - 5)</td>
<td>4 (3 - 5)</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>4</td>
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<td></td>
<td>2</td>
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<td></td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Median (min-max)</td>
<td>5 (4 - 5)</td>
<td>5 (3 - 5)</td>
<td>5 (3 - 5)</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>1</td>
<td>5</td>
<td>5</td>
<td>4</td>
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<td></td>
<td>2</td>
<td>6</td>
<td>5</td>
<td>3</td>
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<td>5</td>
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<td>5</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Median (min-max)</td>
<td>6 (5 - 6)</td>
<td>5 (4 - 6)</td>
<td>4 (3 - 6)</td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>1</td>
<td>5</td>
<td>5</td>
<td>5</td>
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<td></td>
<td>2</td>
<td>6</td>
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<td>3</td>
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<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Median (min-max)</td>
<td>6 (5 - 6)</td>
<td>6 (5 - 6)</td>
<td>6 (5 - 6)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3:** Data for the animals included in the study considering variable movement of the Tarlov scale.
a normal, yet slow gait. In groups G3 and G4 the animals presented a rapid gait without deficiency.

In the period of fourteen days after arterial lesion, groups G1, G2 and G3 presented a normal yet slow gait. Group G4 maintained a fast gait without deficiency.

On the thirtieth post-operatory day, groups G1 and G3 presented a gait with moderate deficiency, and the animals in group G2 presented a normal yet slow gait. G4 presented a rapid normal gait.

Descriptive statistics are presented on the table below according to the groups, evaluation times, and p-values (Table 4).

Since statistical differences were identified on the 14th and 30th days after treatment, the comparison between groups was done two by two.

On the 14th and 30th days there was a difference between G1 and G4, (acute control and chronic treated) suggesting a recovery of motor function in the animals of the chronic ischemia group that received mononuclear stem cell transplants.

In the same period, when comparing groups G2 and G4 (chronic control and chronic transplant) an improvement was identified in symptoms evidenced by the statistical difference between the two groups and suggesting a benefit from treatment.

There were no significant statistical differences found in the other comparisons (Table 5).

Chart 1 identifying the Tarlov scale, in relationship to the scale of movement.

Analysis of variable score of tissue ischemia

Intergroup analysis: Results showed that in the evaluations on the 7th, 14th, and 30th days, all animals of the four groups presented scores of 5, in other words, they presented no degree of tissue ischemia.

Descriptive statistics are presented on the table below according to groups, evaluation times, and p-values for the statistical tests (Table 6).

Analyses of the variable degree of modified tissue ischemia

Intergroup analysis: These results indicated that for the evaluations on the 7th, 14th, and 30 days, all animals’ scores were 7 (no animal presented tissue necrosis of the affected limb).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Evaluation</th>
<th>Group</th>
<th>n</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
<th>P Value (G1×G2×G3×G4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Movement</td>
<td>7 days</td>
<td>G1</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>6</td>
<td>0.122</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G2</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G3</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>G4</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14 days</td>
<td>G1</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>5</td>
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<tr>
<td></td>
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<td>G2</td>
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<td></td>
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<td>G3</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>6</td>
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<td></td>
<td></td>
<td>G4</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>30 days</td>
<td>G1</td>
<td>5</td>
<td>4</td>
<td>3</td>
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<td></td>
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<td>G2</td>
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<td></td>
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<td>G3</td>
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<td>4</td>
<td>3</td>
<td>6</td>
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<td></td>
<td></td>
<td>G4</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td>0.038</td>
</tr>
</tbody>
</table>

Table 4: Descriptive statistics according to groups, evaluation times, considering the variable movement of the Tarlov scale.

<table>
<thead>
<tr>
<th>Groups compared</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14 days</td>
</tr>
<tr>
<td>G1 and G2</td>
<td>0.860</td>
</tr>
<tr>
<td>G1 and G3</td>
<td>0.143</td>
</tr>
<tr>
<td>G1 and G4</td>
<td>0.007</td>
</tr>
<tr>
<td>G2 and G3</td>
<td>0.105</td>
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<tr>
<td>G2 and G4</td>
<td>0.005</td>
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<tr>
<td>G3 and G4</td>
<td>0.143</td>
</tr>
</tbody>
</table>

Table 5: Comparisons between groups considering variable movement.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Evaluation</th>
<th>Group</th>
<th>n</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
<th>p-value (G1×G2×G3×G4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degree of tissue ischemia</td>
<td>7 days</td>
<td>G1</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td></td>
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<td></td>
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<td>G2</td>
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<td>G3</td>
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<td></td>
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<td>G4</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>5</td>
<td>0.284</td>
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<tr>
<td></td>
<td>14 days</td>
<td>G1</td>
<td>5</td>
<td>5</td>
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<td>30 days</td>
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<td>G3</td>
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<td>G4</td>
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</tbody>
</table>

Table 6: Descriptive statistics according to groups, evaluation times, and p-values of the statistical tests in relationship to the variable of tissue ischemia.
In one animal of the group 2, with 14 days of evolution, iliac artery ligation post identifying tissue injury and discoloration of one claw (Figure 2).

The Table 7 below shows descriptive statistics according to the groups, evaluation times, and p-values of the statistical tests.

**Histopathological findings**

In the group 4 that received mononuclear cells, new vessels and endothelial cells were identified, surrounded by collagenous tissue in skeletal muscle as demonstrated by factor VIII immunostaining (Figure 3). In the group 3 that received mononuclear cells, we have not observed the new vessels and endothelial cells (Figure 4).

**Discussion**

Several studies have proven the efficacy of bone marrow mononuclear stem cell transplant in cases of PAOD, utilizing experimental animal models and even clinical studies; however, the results of its effect on acute ischemia of lower limbs have not been well established [6-9].

The clinical evaluation of our proposed experimental model was also challenging, as it was necessary to quantify subjective data and make it as precise as possible. This challenge is inherent for all who must clinically evaluate their patients. Accordingly, we must seek scales for which the objective is to help define clinical conduct, such as the CEAP scale for venous insufficiency, or the *Rutherford* scale for peripheral arterial insufficiency. Fortunately, we identified in the literature some invaluable material regarding the *Tarlov* scale, which was initially developed for evaluating the response to spinal cord injury in rats, and subsequently adapted for motor evaluation in ischemia of lower limbs based on the aforementioned *Rutherford* scale and adapted for the rabbit model. The degree of tissue ischemia score and the modified ischemia score were also utilized to evaluate limb ischemia [5].

The present study evaluated animals with acute and chronic ischemic lesions, separated by a period of 48 hours. All animals presented effectiveness in the lesion achieved by suppression of arterial flow to the lower right limb. The choice of occlusion of the iliac artery and not the right femoral artery was based on the fact that there could be a risk of collateral circulation of the limb, which could thus interfere in the results of the study. Despite this precaution, the presence of collateral vessels was still identified on the *Doppler*. However, they presented very little flow and appeared only after thirty days from time of lesion in all groups.

For the proposed model, all animals were considered homogenous in the pre-transplant period, from both a *Doppler* evaluation and clinical evaluation standpoint.

There was no statistically significant difference between the two
Acute ischemia groups with respect to benefits of cell transplant or movement parameters by the Tarlov scale since in the chronic ischemia groups it was not possible to perform the evaluations due to highly similar values.

With respect to groups G1 and G3, a worsening in movements was identified in both the treated group as well as the control. One of the hypotheses to be discussed involves the fact that in the acute ischemia model, an intense local inflammatory response occurs, that can contribute to the destruction of transplanted cells. In G1, the inflammatory cells could also have contributed to the worsening of the scale values.

The post ischemia inflammatory reaction has been well described in several studies, in which intense proliferation of macrophages both at the lesion site and systemically has been identified. In acute ischemia an intense inflammatory response occurs. These data corroborate with the results identified in the two groups of acute ischemia of the present study [10-18].

With respect to the chronic ischemia group in the intragroup analysis, it was not possible to evaluate the results for either the control or the treated group. However, when we evaluated the absolute values of each animal, an improvement or clinical stabilization was observed, suggesting benefits of the cellular therapy in this group.

In the intergroup analysis, an improvement was identified in the chronic ischemia group that received cells when compared to the control groups and also in comparison to the G1, on the 14th and 30th days after lesion.

In the comparison of animals of group G3 and G4, a tendency was identified with a value of \( p=0.055 \), suggesting that the animals with chronic ischemia that underwent cell transplant presented better results than the acute ischemia group. These data confirm the supposition that the inflammatory response causes lesions on mononuclear bone marrow cells.

A clinical improvement was observed in the chronic ischemia group that received cell transplants. Clinical evaluation was the most effective parameter that we found to evaluate improvement for the animals.

For the degree of tissue ischemia parameter, a clinical inspection of the lower paw was done, observing clear signs of ischemia. On the other hand, the modified ischemia score presented more subtle details that required more attention by the observer. The two parameters are complimentary and thus provide a more complete evaluation with regard to the degree of ischemia of the affected limb.

These data reflect the fact that the animals that received mononuclear stem cell transplant in the chronic phase of ischemia presented better results in comparison to those that did not receive cells in both acute and chronic phases of ischemic lesion.

In the present study, upon evaluation of the clinical criteria for the animals, we did not observe a benefit from the bone marrow cell transplant in the acute phase; however an improvement was seen in the chronic phase, 48 hours after ligation. We believe that this is a result of the intense initial inflammatory response to the acute arterial occlusion leading to transplanted cell death, as well as local cellular metabolic acidosis that does not allow for cellular multiplication.

We believe that for this reason we did not observe an improvement in the acute phase. Transposing these results to a clinical study, we would suggest that cell transplant therapy not be initiated during the first hours after occlusion, or if initiated, treatment be applied in conjunction with therapy to reduce the inflammatory response.

Tateishi-Yuyama et al. in 2002 published a randomized controlled pilot study involving 25 patients with advanced stage chronic local peripheral arterial ischemia who experienced pain at rest and presented unhealed ulcers, or both. These patients due to technical impossibilities were not indicated for arterial revascularization. The patients underwent autologous bone marrow stem cell transplant and an improvement was observed in the ankle-brachial index (ABI), rest pain, and pain free walking time at four and 24 weeks. In the present study we also observed a clinical improvement in the animals that underwent stem cell transplant in the chronic phase [10].

Lawwall et al., in his review article, identified the need for specific animal models with atherosclerotic disease, as it is the principal etiology of peripheral arterial disease in humans. In the present study, we also encountered similar difficulties regarding animal choice. Our model also did not present atherosclerosis, however, was developed to mimic ischemia of the lower limb, a similar consequence between the two primary pathologies [19]. These same authors also referred to a lack of standardization regarding cell types for transplantation and their respective purified fractions to be utilized as angiogenic sites.

In a study by Guarita-Souza et al. two bone marrow cell fractions were compared, mononuclear and mesechymal. In a myocardial infarction model, there was no difference between the two types of transplanted cells with respect to functional effects. For this reason bone marrow mononuclear cells were chosen for this project [20].

The authors also reported that the pathway for administering should be standardized in cases of bone marrow stem cell transplant for chronic ischemia of lower limbs. They suggested that intramuscular

### Table 7: Descriptive statistics according to groups, evaluation times, and p-values in relationship to the variable score of modified tissue ischemia.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Evaluation</th>
<th>Group</th>
<th>n</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
<th>p-value (G1×G2×G3×G4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degree of modified ischemia</td>
<td>7 days</td>
<td>G1</td>
<td>5</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G2</td>
<td>5</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G3</td>
<td>5</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G4</td>
<td>5</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14 days</td>
<td>G1</td>
<td>5</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G2</td>
<td>5</td>
<td>7</td>
<td>4</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G3</td>
<td>5</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G4</td>
<td>5</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>0.392</td>
</tr>
<tr>
<td></td>
<td>30 days</td>
<td>G1</td>
<td>5</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G2</td>
<td>5</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G3</td>
<td>5</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G4</td>
<td>5</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>1</td>
</tr>
</tbody>
</table>
or subcutaneous pathways present the greatest benefit, since cellular loss during injection of the cells is lower and consequently presents better results. Furthermore, according to Pouzet et al. there is a direct relationship between the number of transplanted cells and the functional effect [21].

Nakamuta et al. also presented an opinion in favor of intramuscular injection upon their comparison of three types of stem cell injection: intramuscular, endovenous, and intra coronary, for treatment in cardiac failure. Intramuscular injection of cells was more effective than the others [15].

Miyamoto in an uncontrolled study performed bone marrow mononuclear stem cell transplants in eight patients with PAOD/TAO treating eleven lower limbs in these patients. An improvement was observed in pain at rest, pain scale, and healing of ischemic ulcers. However, in four patients they observed important side effects such as worsening of pain at rest in one patient, worsening of ischemic ulcers in another, sudden death of a 30-year-old patient 20 months after stem cell transplant, and one who presented symptoms compatible to arteriovenous shunt in the calf that appeared 7 months after transplant and worsened after 1 year [22].

This data is contradicted by other studies in the literature, such as Amann et al. [23,24] and the TACT study, for which they carried out more than 100 procedures utilizing bone marrow mononuclear cells and concluded that it is a secure procedure with minimal short and mid-term side effects.

In the present study we did not observe side effects related to the stem cell transplant.

Some studies have suggested different cell types and a variety of autologous and allogeneic tissues as sources, such as dental pulp, adipose tissue, placenta cells, and umbilical cord cells [25-29].

In 2005, Nakagami et al. [30] in an experimental study evaluated the effects of cells derived from adipose tissue on ischemic disease and concluded that it is a secure procedure with minimal short and mid-term side effects.

Study Limitation

The study was not performed for the identification of the mononuclear cells, like as Bromodeoxyuridine (BrdU) or others.

Conclusion

Based on the data of the present study transplant of mononuclear stem cells from bone marrow presents a functional improvement for animals with chronic ischemia in the right lower limb when compared to controls and when compared to those with acute ischemia, both for groups receiving cells and as well as their respective control group.

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Long-term clinical outcome after intramuscular implantation of bone marrow 
mononuclear cells (Therapeutic Angiogenesis by Cell Transplantation [TACT] 

marrow cell transplantation increases leg perfusion and reduces amputations in 
patients with advanced critical limb ischemia due to peripheral artery disease. 

source for vasculogenesis in ischemia: subfraction of side population cells from 

al. (2009) Early translation of adipose-derived cell therapy for cardiovascular 

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mesenchymal stem cells from human placental decidua basalis and resistance 

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