Effects of Autologous Bone Marrow Mononuclear Cells Implantation in Canine Model of Pulmonary Hypertension

Yun Luan, PhD; Zhao-Hua Zhang, PhD; De-E Wei, MD; Yan Lu, MD; Yi-Biao Wang, MD

Pulmonary hypertension (PH) is a refractory blood vessel disorder of the lungs. Pressure in the pulmonary artery (the blood vessel that leads from the heart to the lungs) rises above normal levels and may become life threatening. PH, also known as pulmonary arterial hypertension (PAH), is a condition characterized by an increase in pulmonary vascular resistance (PVR), thrombosis, pulmonary vascular remodeling, structural remodeling of the heart, right heart dysfunction and widespread loss of the pulmonary microvasculature that carries blood from the heart to the lungs. Despite the many treatment options for PAH that have been tested so far, and possibly an improvement in survival, most patients eventually become resistant to therapy and succumb to the disease.

To regenerate healthy normal endothelium, the transplantation of bone marrow cells, such as endothelial progenitor cells (EPC), mesenchymal stem cells and bone marrow-derived mononuclear cells (BM-MNCs) has been documented. It has been shown that autologous transplantation of BM-MNCs could improve left ventricular (LV) function in patients with chronic heart failure and acute myocardial infarction. Yoo et al have showed that transplantation of autologous bone marrow cells has been investigated for angiogenesis and myogenesis in ischemic myocardium.

In the present study, we established a model of PH by intravenous injection of dehydromonocrotaline (DMCT), and then intratracheal administration of autologous BM-MNCs (ABM-MNCs). These processes are thought to decrease the constriction of the pulmonary arteries and their resistance. The purpose of this study was to evaluate the effect of ABM-MNC implantation in DMCT-induced PH dogs and discuss the mechanism.
Cells were washed with PBS and re-suspended in 100μl saline and kept on ice prior to intratracheal injection. Under sterile conditions, the trachea was exposed through a midline incision, and 100μl of cell suspension were injected with a 1-ml syringe as previously described. A total of 1–3×10^7 ABM-MNCs were injected into the trachea 2 weeks after DMCT administration. Cell viability by trypan blue exclusion >95% was required before injection in all experiments.

**DMCT Administration**
DMCT, an active intermediate of monocrotaline, was prepared as described and dissolved in dimethylformamide (0.1 ml/kg; Aldrich, Milwaukee, WI, USA) just before injection. After the baseline hemodynamics were measured, animals were given a single injection of 3 mg/kg DMCT via the right atrium (RA). Following successful establishment of PH, the animals were randomly assigned to 4 groups (n=10 in each group): control group I: dogs that received an injection of 0.9% saline via the RA; group II: dogs that received ABM-MNCs administration (ABM-MNCs group); group III: dogs that received an injection of 3 mg/kg DMCT via the RA (PH group); group IV: dogs that received ABM-MNCs 2 weeks after DMCT injection (PH+ABM-MNC group).

**Hemodynamic Measurements**
Hemodynamic data were recorded 8 weeks after operation as previously described with some modifications. Via femoral vein access, a 5Fr Swan-Ganz catheter (Edwards Lifesciences, Irvine, CA, USA) was advanced into the pulmonary artery for determination of heart rate (HR), central venous pressure (CVP), PVR, systolic pulmonary artery pressure (sPAP), mean pulmonary arterial pressure (mPAP) and pulmonary artery wedge pressure (PAWP). Another catheter was placed in the femoral artery for measurement of systemic arterial pressure (SAP).

**Immunological and Immunohistochemical Analyses**
Dogs were euthanased after the hemodynamic measurements, and the lung and heart were quickly harvested and fixed in situ via the tracheal cannula with buffered 10% formaldehyde, then embedded in paraffin. The sections were cut into 5-μm slices and stained with streptavidin peroxidase and hematoxylin-eosin (H&E; Baso Biotechnology, Shenzhen, China). The morphometric analysis of the pulmonary arteries was as described previously. Briefly, the percent of muscular arteries (MA%) and partially muscular arteries (PMA%) with an external diameter of 15–50 μm at the alveolar wall and alveolar duct levels were measured and calculated at ×400 magnification. The percent medial wall thickness (%MWT) of muscular

### Methods

#### Animals
Forty healthy adult mongrel dogs weighing 10–15 kg were purchased from the animal experimental center of Shandong University, China. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health (NIH Publication No. 85-23, revised 1996).

#### ABM-MNCs Preparation and Intratracheal Injection
The preparation of the ABM-MNCs was carried out according to previous studies. Under general anesthesia, bone marrow (20 ml) was aspirated from the greater tubercle of the humerus with a myeloid puncture needle and a syringe containing 6,000U heparin. Ficoll density gradient centrifugation was used to separate ABM-MNCs and other cells. The white coat composed of mononuclear cells from the upper layer and interface was carefully collected and washed 3 times before final re-suspension in 10 ml heparinized saline. Cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-CD34 monoclonal antibodies (BD Biosciences, San Jose, CA, USA), and the percentage of CD34-positive cells was determined by fluorescent-activated cell sorting (FACS) analysis. After the cells were washed 3 times in phosphate buffered saline (PBS), they were labeled with the cross-linkable membrane dye (CM-DiI; 2 μg/ml, Invitrogen Corp, CA, USA).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Tm (°C)</th>
<th>Cycles</th>
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</thead>
<tbody>
<tr>
<td>GAPDH</td>
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<tr>
<td></td>
<td>5'-CAGGATACCCCCCATTTGATG-3'</td>
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<td>VEGF</td>
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<td>35</td>
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<tr>
<td></td>
<td>5'-ACTCCTGGAGATGTCCCA-3'</td>
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</tr>
<tr>
<td>ppET-1</td>
<td>5'-AGCACTGGTGCGAGGAA-3'</td>
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<td>35</td>
</tr>
<tr>
<td></td>
<td>5'-CTCGTTTCTACGCCAACA-3'</td>
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<td>IL-6</td>
<td>5'-GAACCTCTCCCTCCACAACG-3'</td>
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<td>35</td>
</tr>
<tr>
<td></td>
<td>5'-TCTGTCAAGCAGTCTCC-3'</td>
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<tr>
<td>TNF-α</td>
<td>5'-GCCCAGACAGATCAATCATCT-3'</td>
<td>52</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>5'-CTTGTAGGCGAGAGATGTT-3'</td>
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</table>

**VEGF, vascular endothelial growth factor; ppET-1, preproendothelin-1; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α.**

![Cross-linkable membrane dye labeled autologous bone marrow-derived mononuclear cells showing labeling efficacy >90% (x100).](image)
Table 2. Effect of ABM-MNCs on Hemodynamics and the RV

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (beats/min)</td>
<td>145±16</td>
<td>143±14</td>
<td>147±10</td>
<td>146±12</td>
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<tr>
<td>CVP (mmHg)</td>
<td>3.42±0.63</td>
<td>3.61±0.34</td>
<td>4.06±0.45</td>
<td>3.81±0.22</td>
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<td>SAP (mmHg)</td>
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<td>140.6±3.27</td>
<td>146.7±4.5</td>
<td>145.4±3.4</td>
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<td>sPAP (mmHg)</td>
<td>21.2±1.8</td>
<td>20.7±3.8</td>
<td>56.2±7.8*</td>
<td>38.4±5.1*</td>
</tr>
<tr>
<td>mPAP (mmHg)</td>
<td>12.5±2.1</td>
<td>12.1±1.2</td>
<td>38.5±6.4*</td>
<td>24.7±3.7*</td>
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<tr>
<td>PVR (dyne·s·^{-1}·cm^{-2})</td>
<td>346.8±51</td>
<td>392.0±40</td>
<td>2,194.3±168*</td>
<td>1,995.0±134*</td>
</tr>
<tr>
<td>PAWP (mmHg)</td>
<td>8.3±3.06</td>
<td>8.24±3.14</td>
<td>17.0±1.08*</td>
<td>14.13±1.36*</td>
</tr>
<tr>
<td>RV/(LV+S)</td>
<td>0.34±0.08</td>
<td>0.36±0.07</td>
<td>0.48±0.05*</td>
<td>0.42±0.06*</td>
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<tr>
<td>CO (L/min)</td>
<td>3.75±1.38</td>
<td>3.62±1.05</td>
<td>2.57±1.47*</td>
<td>3.26±0.12*</td>
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</tbody>
</table>

The data are mean±SE (n=10).

*P<0.05 compared with control group; **P<0.05 compared with group III.

ABM-MNCs, autologous bone marrow-derived mononuclear cells; RV, right ventricle; HR, heart rate; CVP, central venous pressure; SAP, systemic arterial pressure; sPAP, systolic pulmonary artery pressure; mPAP, mean pulmonary arterial pressure; PVR, pulmonary vascular resistance; PAWP, pulmonary artery wedge pressure; RV/(LV+S), right ventricular/left ventricular plus septal weight; CO, cardiac output.

I: negative control group; II: ABM-MNC group; III: pulmonary hypertension (PH) group; IV: PH+ABM-MNC group.

arteries with an external diameter of 100–200 μm was calculated with KS400 image analysis software. After the hearts were removed, the right ventricle (RV) was separated from the LV plus the septal wall (S), and both parts were weighed to assess RV hypertrophy (RVH). The ratio of the RV free wall to the LV+S weight [RV/(LV+S)] was determined to measure RVH.

The degree of angiogenesis was determined by the density of pulmonary arterioles measured in the lung tissue by light microscopy according to the Weidner method. Briefly, after staining the sections with monoclonal rabbit antibodies CD34 (1:100, BD Biosciences), 5 non-overlapping “hotspots” where the number of capillaries was at a maximum in transverse sections were captured at low magnification (×100), and the number of brown-yellow capillaries (CD34-positive) was counted in each of the 5 hotspots at high magnification (×200). The density was counted in 50 sections by 2 examiners who were blinded to treatment assignment.

Real-Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) Analysis

After the lungs were harvested, total RNA was extracted from lung tissue homogenates (Invitrogen). qRT-PCR analysis was performed to detect the relative pulmonary expression levels of vascular endothelia growth factor (VEGF), preproendothelin-1 (ppET-1), interleukin (IL)-6 and tumor necrosis factor (TNF)-α. The RNA sample was dissolved in RNase-free water and quantified spectrophotometrically. Primers were designed using the Primer Express software package (Applied Biosystems) and are shown in Table 1. Data were analyzed with the ABI Prism 7900 sequence detection system software (version 2.2) and β-actin was used as an internal control for input RNA.

Identification of the Intratracheal Injected Cells

Transplanted ABM-MNCs were examined by observing the presence of CM-Dil labeled cells under fluorescent microscopy. The freshly excised lungs were embedded in optical coherence tomography compound (Sigma-Aldrich, St Louis, MO, USA), quickly frozen in liquid nitrogen and stored at −80°C. The sections were cut into 4-μm slices and incubated with primary antibodies after being fixed in acetone for 10 min at 4°C. Transformation of engrafted cells was shown by rabbit anti-human von Willebrand factor (vWF, Abcam, Cambridge, UK) antibody, α-smooth muscle actin (SMA, Santa Cruz Biotechnology, CA, USA) antibody and surfactant associated protein C (SP-C, Santa Cruz Biotechnology) antibody according to each manufacturer’s recommendations. The fluorescent-positive sections were incubated with FITC-conjugated secondary antisera (Abcam). After washing with PBS, the sections were observed and photographed under fluorescent microscopy.

Statistical Analysis

Statistical analyses were performed with 1-way analysis of variance followed by Bonferroni test or t-test when appropriate (SPSS version 13.0, Chicago, IL, USA). Differences were considered significant at P<0.05 and data are means±SE unless otherwise stated.

Results

Mortality in Each Group

No deaths occurred within groups I, II and IV during the study period, but 2 dogs in group III died of acute pulmonary edema within the first week of DMCT administration; 2 replacement dogs underwent the same procedure and treatment as in group III. Therefore, 10 dogs in each group were available for analysis.

Fluorescence-Activated Cell Sorting Analysis and Fluorescence Staining

In the present study, FACS showed that the lymphomonocytic fraction (CD34+) was 1.8±0.3% by density gradient centrifugation and the labeling efficiency by CM-DiI reached levels as high as 90%, as observed in vitro under a fluorescent microscope before transplantation (Figure 1).

Effect of ABM-MNCs on Hemodynamics and the Right Ventricle

There were no significant differences in initial HR, CVP, SAP, PVR, sPAP, mPAP or PAWP among the 4 groups. At 8 weeks after operation, PVR, sPAP, mPAP and PAWP did not differ between groups I and II, but were significantly higher in groups III and IV (P<0.05). Furthermore, PVR, sPAP, mPAP and PAWP were remarkably lower in group IV than in group III (P<0.05) (Table 2). HR and CVP remained relatively stable throughout the study period. After the animals were anesthetized, the hearts were removed and weighed, the RV was separated from the LV+S. Our results showed that the RVs were enlarged and RV/(LV+S) weight ratio was significantly
Figure 2. Effect of autologous bone marrow-derived mononuclear cells (ABM-MNCs) on pulmonary artery wall 8 weeks after operation: (H&E staining demonstrates intimal thickening in group III compared with groups I and II after injection of 3mg/kg dehydromonocrotaline, but improvement in group IV compared with group III 6 weeks later (×400, black arrows, A). Comparison of the value in 4 groups: the percent of muscular arteries (MA%) and partially muscular arteries (PMA%) among muscular arteries with an external diameter of 15–50μm was significantly increased in group III as compared with groups I and II, and significantly decreased in group IV compared with group III (P<0.05, B, C). Percentage medial wall thickness (%MWT) of muscular arteries with an external diameter of 100–200μm was significantly increased in group III compared with groups I and II, and decreased in group IV compared with group III (P<0.05, D). The data are mean±SE (n=10). *P<0.05 vs. control group; #P<0.05 vs. group III. I: negative control group; II: ABM-MNC group; III: pulmonary hypertension (PH) group; IV: PH+ABM-MNC group.
higher in group III compared with the control (P<0.05), and lower in group IV than in group III (P<0.05). There was no difference between groups I and II (P>0.05) (Table 1).

Effect of ABM-MNCs on Pulmonary Artery Wall and Vessel Density

At 8 weeks after DMCT injection, H&E staining demonstrated intimal thickening in group III compared with groups I and II, which improved in group IV compared with group III (Figure 2A). MA% and PMA% of muscular arteries with an external diameter of 15–50μm were significantly increased in groups III and IV as compared with groups I and II (P<0.05). Furthermore, these were more significantly decreased in group IV than group III (P<0.05, Figure 2B). We also analyzed the %MWT for 4 groups of muscular arteries with an external diameter of 100–200μm and our results showed that the %MWT was more significantly increased in groups III and IV than in groups I and II, but more decreased in group IV than in group III (P<0.05, Figure 2C).

The capillary density of pulmonary arterioles was detected by histological staining (CD34) in the lungs (Figure 3A), and the results showed that the density was significantly decreased in group III compared with groups I and II (P<0.05). More importantly, it was significantly increased in group IV compared with group III (P<0.05, Figure 3B).

mRNA Expression in Pulmonary Tissue

RT-PCR results showed that there was no difference between...
groups I and II in the mRNA expression of VEGF, ppET-1, IL-6 and TNF-α in the lungs. The expression of VEGF (2.06±0.43) in the lungs was significantly lower, and that of ppET-1, IL-6 and TNF-α significantly higher in group III compared with groups I and II, and were significantly improved in group IV compared with group III (P<0.05). The data are mean±SE (n=10). *P<0.05 vs. control group; #P<0.05 vs. group III. I: negative control group; II: ABM-MNC group; III: pulmonary hypertension (PH) group; IV: PH+ABM-MNC group.

**Discussion**

This study demonstrated that intratracheal injection of bone marrow-derived mononuclear cell is safe and feasible. Our results suggest that 6 weeks after injection of ABM-MNCs into a canine model of PH the following occurred: (1) hemodynamic data were significantly improved; (2) the RV/(LV+S) weight ratio was significantly lower; (3) arteriolar narrowing and alveolar septum thickening were marked reduced; and (4) the cells could survive and differentiate into vascular endothelial cells in vivo and angiogenesis in lung were significantly enhanced.

PH occurs when most of the very small arteries throughout the lungs become narrow in diameter, which increases the resistance to blood flow. PH often occurs as a consequence of an isolated pulmonary arteriolar vasculopathy, and is then called PAH,19 which is characterized by functional and structural changes in the pulmonary vasculature, leading to increased PVR, RV dysfunction,20 lung vascular remodeling and loss of the distal pulmonary vasculature.21 There are a large number of therapies that have been proven useful in decreasing pulmonary arterial pressure, improving exercise tolerance and quality of life, but an effective therapy with a long-term outcome is lacking.18,22 In recent years, studies have shown that endothelial dysfunction is an inevitable feature and an early event in the pathogenesis of PH.8,23 As reported, inflammatory mechanisms...
ABM-MN and PH Therapy

also play a significant role in MCT-induced PAH, such as IL-1, IL-6, transforming growth factor-β. Growth factors have been proven to have a vasodilatory effect on the vasculature, and VEGF has been shown to cause pulmonary vasodilation.

For these reasons, regenerative methods and gene therapy have been drawing considerable attention to the breaking of the vicious cycle of PH. Bone marrow-derived mononuclear cells isolated from bone marrow, including stromal cells, vascular cells, mesenchymal stem cells and hematopoietic stem cells, have the ability to differentiate into EPCs and vascular endothelial cells in vitro. Transplanted ABM-MNCs could augment neovascularization and incorporate into the capillary endothelial cell network in ischemic tissues. Previous studies have demonstrated that BM-MNC release multiple growth factors, including VEGF, stromal cell derived factor-1, insulin-like growth factor, and platelet-derived growth factor. Currently, transplantation of EPCs into MCT-induced PAH models improves the impairment, but reports about BM-MNC transplantation for treatment of PH are few. ABM-MNCs have been the most commonly used cell preparation in clinical studies.

MCT, a pyrrolizine alkaloid extracted from the seeds of Crotalaria spectabilis, is converted by the mixed-function oxidase system of the liver into DMCT. The MCT-induced PH model is known to present similar pathology to that of primary PH. In the present study, 2 weeks after well-established PH by intravenous injection of 3 mg/kg DMCT via the RA, we intratracheally administered 1–3×10^7 labeled ABM-MNCs and investigated the effect on DMCT-induced PH 6 weeks later. We found that the injection of ABM-MNCs into dogs with PH had beneficial effects on the lungs and heart, such as the hemodynamic data (PVR, sPAP, mPAP and PAWP), which were significantly improved, and the RV/(LV+S) weight ratio, which was significantly lower in the ABM-MNCs group compared with the PH group. In the present study, PAWP was remarkably lower in the PH+ABM-MNCs group than in the PH group, which means LV function or preload was significantly improved. The mechanism may relate to the ability of ABM-MNCs to improve the hemodynamic changes of end-stage PH, protect against DMCT-induced endothelial injury and increase the blood or oxygen supply. None of these could be clarified in the present study and would need further investigations.

Inflammatory mechanisms play a significant role in PH, as in the MCT model in the rat, where the primary importance of inflammation is illustrated by successful therapies using an IL-1 receptor antagonist and antibodies to monocyte chemoattractant protein-1. Endothelial dysfunction is also common in PH, and includes increased production of vasoconstrictor/mitogenic compounds such as ET-1. DMCT-induced endothelial cell dysfunction is associated with deregulated expression of proinflammatory mediators that may cause these changes.

The present study showed significantly increased pulmonary expressions of ppET-1, IL-6 and TNF-α in the DMCT-induced PH group compared with the control; these levels were significantly decreased in the ABM-MNCs group compared with the PH group. These results indicate that intratracheal administration of ABM-MNCs has a protective effect against DMCT-induced PAH.
induced endothelial damage in this canine model of PH.

On the other hand, analysis of the histology results in 4 cases showed intimal thickening in the DMCT-induced PH group compared with control, but it improved in the ABM-MNCs group compared with the PH group. MA% and PMA% for muscular arteries (15–50-μm external diameter) and %MWT for muscular arteries (100–200-μm external diameter) were significantly decreases in the ABM-MNCs group as compared with the PH group. Based on these results, we hypothesize that the mechanism of inhibition of intimal and medial wall thickening of ABM-MNCs may be related to their antiinflammatory effects.

The immunohistochemical staining of lung sections showed that the intratraheal injected ABM-MNCs could survive for more than 6 weeks and cells in lung labeled positive for CM-Di (red fluorescence) also stained positive for vWF and SMA. The merged images for CM-Di and for vWF and SMA staining, represented by a yellow color, suggested that transplanted ABM-MNCs retain expression of markers specific for endothelial and smooth muscle cell phenotypes.\textsuperscript{10,11} On the other hand, studies have shown that bone marrow-derived mesenchymal stem cells (BMSCs) produce growth factors such as VEGF that promote neovascularization, which participates in BMSCs-based therapies for PH.\textsuperscript{20} In fact, our qRT-PCR results demonstrated that the relative expression concentration of VEGF (2.06±0.43) in the lungs was significantly improved in the ABM-MNCs group (4.53±0.75), and the histological results showed that the density of pulmonary arterioles was significantly increased in the ABM-MNCs group compared with the PH group. However, red fluorescent-positive cells stained negative for SP-C, suggesting intratraheal injected ABM-MNCs are able to differentiate into vascular endothelial cells, but not lung cells, in vivo. These results showed that injected ABM-MNCs, by angiogenic and vasculogenic mechanisms, may improve endothelial function by releasing factors. The present study for the first time has demonstrated that ABM-MNCs can differentiate into vascular endothelial cells and smooth muscle muscle cells in vivo, although they did not actively survive as lung cells 6 weeks after intratraheal injection into a canine model of PH.

Study Limitations

There are several possible mechanisms by which ABM-MNCs suppressed intimal and medial wall thickening in this canine model of PH, including induction of pulmonary artery wall apoptosis, inhibition of pulmonary artery smooth muscle cell proliferation, and/or antiinflammatory effects. None of these could be clarified in the present study and would need further investigations.

Conclusions

Intratraheal administration of ABM-MNCs may improve the impairment of lung and heart caused by DMCT-induced PH, decrease pulmonary arterial narrowing and enhance angiogenesis by differentiating into pulmonary vascular endothelial cells. The mechanisms include antiinflammatory, angiogenesis/vasculogenesis and paracrine mediator effects.

Acknowledgments

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Disclosures

Conflict of Interest: None declared.

References


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