The effect of combination of low molecular heparin and Galectin-3 on the cell migration and cell proliferation of vascular endothelial cell derived from mesenchymal stem cells

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Abstract: Objective: To explore the effect of the combination of low molecular heparin and Galectin-3 on migration and proliferation of vascular endothelial cell derived from mesenchymal stem cells. Methods: In the present study, there were four groups: low molecular weight heparin group, in which 20 ug/ml low molecular weight heparin was added; Galectin-3 group, in which 5 ug/ml Galectin-3 was added; combination group, in which 20 ug/ml low molecular weight heparin and 5 ug/ml of Galectin-3 was added; control group, in which equal volume of PBS buffer was added. Then we explored the effect of the combination of low molecular heparin and Galectin-3 on migration and proliferation of vascular endothelial cell derived from mesenchymal stem cells. Results: The OD₄₉₀ value of LMWH group is 0.285 ± 0.018, the OD₄₉₀ value of Galectin-3 group is 0.297 ± 0.041, the OD₄₉₀ value of the combined group is 0.351 ± 0.016, and the OD₄₉₀ value of control group is 0.233 ± 0.005, which indicates that the combined treatment could increase the cell proliferation significantly (P < 0.05). After 24 hours culture, the cell migration rate was 42.02 ± 7.62 in low molecular weight heparin group, 45.82 ± 3.96 in Galectin-3 group, 68.53 ± 11.22 in the combined group, and 34.21 ± 3.99 in the control group, respectively, which suggested that combined administration induced the largest cell migration (P < 0.05). Conclusion: The combination of low molecular heparin and Galectin-3 could significantly improve migration and proliferation of vascular endothelial cell derived from mesenchymal stem cells.

Keywords: Low molecular heparin, Galectin-3, vascular endothelial cell, cell migration, cell proliferation

Introduction

With the aging tendency of society, the incidence of chronic peripheral arterial disease (PAD) increases year by year. PAD seriously affects patient’s physical health and life quality [1-3]. Among various types of PAD, arterial occlusive disease of low extremity and diabetic foot are most harmful and difficult to cure [4, 5]. These diseases can lead to limb ischemia in patients, thus it may cause limb necrosis, eventually amputation, and even death [6, 7]. At present, these diseases have received widespread attention. It was reported that stem cell transplantation can be adopted to treat PAD [8]. One study shows that transplanted bone marrow mesenchymal stem cells (MSCs) may differentiate into vascular endothelial cells and smooth muscle cells, thereby repairing the damaged tissue [9]. At the same time, in patterns of autocrine and paracrine, it synthesizes and secretes vascular growth factor to promote angiogenesis. Another study shows that bone marrow MSCs can promote cell proliferation, suppress apoptosis, and promote angiogenesis through its anti-inflammatory effect. In the process of stem cell transplantation vascular endothelial cell is a key factor. Vascular endothelial cells lie between blood vessel wall and blood to form a shield. It is the sole anti-thrombotic cell type in human body [10-12]. In addition, it can produce multiple active substances to protect blood vessel [13-15]. At present, in the study of this field, how to improve the migration and proliferation of MSCs-derived vascular endothelial cells has been a focus.
Heparin is commonly used as anticoagulant drugs clinically to prevent post-operative thrombosis [15-18]. Low molecular weight heparin (about 5 kd) is generated through hydrolysis of heparin mainly. It has been widely used in clinical practice due to its various advantages, such as high efficiency, ineligible affinity to platelet and better stability. Galectin-3 is a kind of glycoprotein. Depending on its glycol-domain, it specifically binds to intracellular glycoproteins, cell surface molecules, glycosylated extracellular matrix proteins and membrane proteins via the lectin-glyco-interaction thus being involved in a series of physiological and pathological processes, including cell growth, apoptosis, cell adhesion, vascularization, tumor invasion, and metastasis [19-21]. Specifically, previous studies show that Galectin-3 may be involved in vascular endothelial cell migration, chemotaxis, angiogenesis and tumor angiogenesis in endothelial cells.

This study had studied the influence of low molecular weight heparin combined with Galectin-3 on the migration and proliferation of vascular endothelial cells derived from bone marrow mesenchymal stem cells, in order to explore potential methods to improve migration and proliferation of vascular endothelial cells in stem cell transplantation.

**Materials and methods**

**Experimental materials**

The experimental animals were 20 SD rats, male, body weight 130-160 g, and the cultured grade was SPF. MTT, RNA enzyme, Galectin-3 were purchased from Sigma company. This study was approved by the ethics committee of Anhui medical university.

**Cell culture**

*Isolation and culture of MSCs:* The rats were executed in sterile conditions, and the cells were collected and inoculated in the 100 mm
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petri dish with L-DMEM culture medium. The cells were cultivated at 5% CO\textsubscript{2} under 37°C. After 2 days, the culturing medium was replaced with new medium. Dead cells and non-adherent cells were discarded. Then the cells were continually cultured under the same conditions, with replacing medium and removing dead cells every 3 days. When cell confluence reaches 80%, the supernatant was discarded, and the cells were washed with PBS repeatedly. Trypsin was added. After 2 to 3 minutes, the reaction was terminated by adding culture medium. The cells were pipetted repeatedly, and then centrifuged at 1000 r/min for 5 min. Supernatant was discarded and the cells were inoculated in L-DMEM culture.

Subculture of cells

After 12 to 14 days culture, the cell confluence reaches 80%. The culture medium was discarded. Cells were washed with PBS for 3 times. 0.125% trypsin was added for digestion for 2~3 minutes. The cells were observed under fluorescence microscope. When the structural cells became irregular, gap junctional intercellular enlarged, L-DIVIEIVI medium was added to terminate the digestion. The cells were cultured and passaged by the ratio of 1:2.

Induction differentiation of MSCs: The cells of passage 4 were selected to test, whose is featured with stablization. 10 ng/ml endothelial growth factor (vascular endothelial growth factor, VEGF) and 2 ng/ml alkaline fibroblast growth factor were added to cells, co-cultured with basic fibroblast growth factor, bFGF). The morphologic change of cells was constantly observed under Light microscope.

Identification of induced cells: Immunofluorescence staining was adopted to observe the expression of vWF. The passage 2 cells of experimental group and the control group were plated in 6-pore plates with built-in slides. Primary antibody (rabbit anti-rat vWF, 1:40) were added and incubated at 4°C overnight. Then plates were washed with PBS, and added with secondary antibody (goat anti rabbit FITC - resistant IgG, 1:10). Plates was washed with PBS, mounted with glycerol and immediately observed under the fluorescence microscope.

TEM observation of induced cells: Cells of 14 days differentiation were selected, washed with PBS, transferred to 15 ml plastic centrifuge tube, and centrifuged under room temperature. The cells were precipitated with 4% paraformaldehyde, 2.5% glutaraldehyde, and fixed at 4°C for 2 hours. The cells were dehydrated with gradient ethanol. Semi thin sections were stained with azure-methylene blue, and located under light microscope; ultrathin slices were stained with uranyl acetate and lead citrate, and observed under electron microscope.

Vascular endothelial cell proliferation and migration

4-methyl thiazole blue nitrogencolorimetry (MTT) colorimetry detecting vascular endothelial cell proliferation: The cells in logarithmic growth phase were selected and plated in 96-well plates, with 1×10\textsuperscript{5}/L cells in each pore. After 24 hours when all cells were adherent to the wall, medium was completely replaced with DMEM medium without serum and cells were continually cultured for 12 hours. The cultured cells were divided into four groups: low molecular heparin group, treated with low molecular heparin to a final concentration of 20 ug/ml; Galectin 3 groups, treated with Galectin-3 to final concentration of 5 ug/ml; combination group, treated with low molecular heparin to final concentration of 20 ug/ml and 5 ug/ml Galectin 3; control group, treated with PBS buffer of the same volume. Cells of all groups were cultured for another 24 hours. Each pore was added with 150 ul dimethyl sulfoxide and plates were laid on shaking table at low speed for 10 min to make the crystal dissolves completely. ELISA (enzyme-linked immunosorbent assay) was adopted to test the absorbance (OD) at 490 nm. Within the scope of a certain number of cells, the value determined by MTT crystallization is proportional to the number of cells. He relative number of living cells and vitality were determined with the measured absorbance

<table>
<thead>
<tr>
<th>Groups</th>
<th>Experiment numbers</th>
<th>OD\textsubscript{490}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low molecular heparin</td>
<td>3</td>
<td>0.285 ± 0.018\textsuperscript{a}</td>
</tr>
<tr>
<td>Galectin-3 group</td>
<td>3</td>
<td>0.297 ± 0.041\textsuperscript{a}</td>
</tr>
<tr>
<td>Combination group</td>
<td>3</td>
<td>0.351 ± 0.016\textsuperscript{a,b,c}</td>
</tr>
<tr>
<td>Control group</td>
<td>3</td>
<td>0.233 ± 0.005</td>
</tr>
</tbody>
</table>

\textsuperscript{a}P < 0.05 vs control group, \textsuperscript{b}P < 0.05 vs low molecular heparin group, \textsuperscript{c}P < 0.05 vs combination group.
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Detection of vascular endothelial cell cycles by flow cytometry

Cell culturing and grouping methods were shown as in the above. The cells were continually cultivated for 24 hours, digested after collection, washed with PBS repeatedly, and diluted into density of $2 \times 10^6$ cells/L. The cells were centrifuged at 1000 r/min for 5 min and washed with PBS. In dark place, after 1 ml of DNA dyeing was added, the cells were repeatedly pipetted and blended. 15 min later, the endothelial cell cycle was tested and calculated with flow cytometry.

Endothelial cell migration analysis: wound healing assay

Cell culture and the grouping situation were shown as in above. Evenly crossed in a petri dish using Marker pen, the cells were covering the bottom of the dish next day. Crossed/Using small white point perpendicular to the bottom of the scratch, PBS wash three times. No-sticking cells and exfoliate cell were discarded. Real-time shooting the dynamic situation of vascular endothelial cell migration for 24 hours. Continuously shoot wound location, analyzes the healing situation.

Statistical analysis

$X^2$ test was used as count data, and analysis of variance was used as measurement data. The measurement results was expressed as a mean $\pm$ standard deviation (+- S). SPSS20.0 software was adopted. Variance Analysis was adopted for comparison between groups. $P < 0.05$ or $P < 0.01$ was regarded as statistically significant.

Results

Subculture of MSCs

The subculture cells grew fast that adherences completed within 24 hours, and integration completed within 4-5 days. The cells showed a uniform long spindle. They showed a neatly brush when growing in group, and showed a spiral pattern in vigorous growth period (Figure 1).

Results of cell differentiation induced by MSCs

One day after induction, the cells became wider, shorter, and were present as polygon; 3 days after induction, the cells stretched out pseudopodia and interconnected with each other; 7 days after induction, the cells were present in funicular; 20 days after induction, the cable structure became longer, like a vessel change, and there were paving stone-like endothelial cells partially (Figure 2).

Immunofluorescence staining and observation of induction cells under electron microscope

Immunofluorescence staining results: The immunofluorescence staining result of induction cells was positive, in which cytoplasm of cells was yellowish green with clear contour (Figure 3); the immunofluorescence staining result of control cells was negative.

Electron microscope results: The cytoplasm was abundant in the induction cells, having more mitochondria and golgi complexes and pinocytosis vesicles, and more rough endoplasmic reticulum and ribosomes scattered in. Thetypical Weibel-Palade (W-P) was seen in part of the cells (Figure 4).

Absorbance (OD$_{490}$) results

Within the scope of a certain number of cells, the value determined by MTT crystallization

### Table 2. The effect of each group on the changing of endothelial cell cycle ($\bar{x} \pm s$, n=3)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Experiment numbers</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
<th>PI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low molecular heparin</td>
<td>3</td>
<td>65.58 ± 1.65$^a$</td>
<td>35.12 ± 0.97$^a$</td>
<td>4.04 ± 0.83$^a$</td>
<td>36.81 ± 1.45$^a$</td>
</tr>
<tr>
<td>Galectin-3 group</td>
<td>3</td>
<td>67.32 ± 1.22$^a$</td>
<td>33.76 ± 1.76$^a$</td>
<td>3.92 ± 0.66$^a$</td>
<td>34.66 ± 1.08$^a$</td>
</tr>
<tr>
<td>Joint group</td>
<td>3</td>
<td>43.15 ± 2.65$^{b,c}$</td>
<td>54.27 ± 1.43$^{b,c}$</td>
<td>5.97 ± 0.38$^{b,c}$</td>
<td>56.01 ± 0.78$^{b,c}$</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>76.79 ± 2.77</td>
<td>29.53 ± 2.97</td>
<td>1.72 ± 0.46</td>
<td>22.85 ± 1.98</td>
</tr>
</tbody>
</table>

$^a$P < 0.05 vs control group, $^b$P < 0.05 vs low molecular heparin group, $^c$P < 0.05 vs combination group.
was proportional to the number of cells. The relative number and vitality of living cells were determined with the measured absorbance value (OD value). The larger the OD value is, the stronger the cell activity will be and the bigger the quantity will be. First, we recorded the absorbance (OD_{490}) data of each sample. Each experiment was repeated three times. As shown in Table 1, the OD value was biggest in combination group, which indicated that under the effect of low molecular heparin combined with Galectin 3, vascular endothelial cell obviously proliferated fastest (P < 0.05). On the other hand, low molecular heparin and Galectin-3 alone also can improve endothelial cells separately (P < 0.05) (Table 1).

![Figure 5](image)

**Figure 5.** Differentiated endothelial cell cycle detected by flow cytometry, which were exposed to different concentrations for 24 h. A: Control group; B: Low molecular heparin group; C: Galectin-3 group; D: Joint group.

### Endothelial cell cycle changing results

The flow cytometry was adopted to analyze endothelial cell cycle changing. Each experiment was repeated three times, to record the different cycles. As shown in Table 2, the proportion of G0/G1 cells in the combination group was the lowest, significantly lower than that in control group (P < 0.05). In addition, the percentage of cells in S phase was highest in the combination group, significantly higher than that in the control group (P < 0.05). Combination group has the highest percentage of cells in G2/M phase, significantly higher than that of control group (P < 0.05). The proliferation index of combination group was significantly higher.
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than that of other groups ($P < 0.05$) (Figure 5; Table 2).

The effect of treatment on the endothelial cell migration distance

Then we detected the number of vascular endothelial cells and cell migration distance under the effect of different treatment. Each experiment was repeated three times. As shown in Table 3, the number of cells in combination group was the biggest, and the maximum distance was the longest, which indicated that under the action of low molecular heparin combined with Galectin 3, the number of vascular endothelial cell and cell migration distance increased significantly faster ($P < 0.05$). In addition, the low molecular heparin or Galectin-3 alone can also promote endothelial cell number and cell migration distance ($P < 0.05$) (Figure 6; Table 3).

**Discussion**

With the aging of society, the incidence of chronic peripheral arterial disease (PAD) increases year by year. PAD seriously affects patient’s physical health and life quality. Among various types of PAD, arterial occlusive disease of low extremity and diabetic foot are the most serious. Therefore, these diseases have aroused widespread attention. It is reported that stem cell transplantation can be adopted to treat PAD. However, some problems in its practical application remain unsolved. A critical one is how to increase the migration and proliferation of vascular endothelial cells. The present methods are costive and inefficient, there is no single surface marker, but rather a panel of surface markers which define MSCs. As per the international society for cellular therapy guidelines, MSCs must express CD105 (SH2), CD73 (SH3/4), and CD90 and must be negative for surface markers CD34, CD45, CD14 and HLA-DR [22]. Detailed phenotypic expression of surface markers is reviewed in this article [23]. Therefore, how to improve the migration and proliferation of MSCs-derived vascular endothelial cells has been the focus of clinical research in this field.

This study selected low molecular heparin and Galectin 3 as the research objects. The heparin is the commonly used anticoagulant drugs in clinical, widely applied in postoperative prevention of thrombosis. Low molecular weight heparin (about 5 kd) is generated through hydrolysis of heparin mainly. It has been widely used in

![Figure 6. Effects of different time points on cell wound healing.](image)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cell number</th>
<th>Maximum distance (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low molecular heparin</td>
<td>28.83 ± 2.25$^a$</td>
<td>402.04 ± 62.52$^a$</td>
</tr>
<tr>
<td>Galectin-3</td>
<td>32.98 ± 3.29$^a$</td>
<td>399.18 ± 63.39$^a$</td>
</tr>
<tr>
<td>Joint group</td>
<td>44.27 ± 5.29$^{a,b,c}$</td>
<td>585.65 ± 44.52$^{a,b,c}$</td>
</tr>
<tr>
<td>Control</td>
<td>12.32 ± 1.21$^a$</td>
<td>241.10 ± 53.26</td>
</tr>
</tbody>
</table>

$^a P < 0.05$ vs control group, $^b P < 0.05$ vs low molecular heparin group, $^c P < 0.05$ vs combination group.
First, this study analyzed the effect on vascular endothelial cell proliferation. In the test to detect endothelial cell number, the results indicated that low molecular heparin combined with Galectin 3 can significantly promote the proliferation of vascular endothelial cells (P < 0.05), compared to single treatment of low molecular heparin or Galectin 3 (P < 0.05). It also suggested that the mechanisms of low molecular heparin and Galectin-3 to promote vascular endothelial cell proliferation are different. The two are a collaborative relationship. Galectin 3 as a binding protein of IgE, can stimulate the tube cavity structure of vascular endothelial cells derived from the bone marrow mesenchymal stem cells. Thus more endothelial cells were produced, so as to reach the requirement for clinical effect. Low molecular heparin improves the endothelial cell proliferation by improving endothelial cell adhesion rate. With two different mechanisms, combination of low molecular heparin and Galectin 3 effectively promote the vascular endothelial cell proliferation rate through synergy.

In conclusion, low molecular heparin combined with Galectin 3 can significantly increase the migration and proliferation of vascular endothelial cells derived from bone marrow mesenchymal stem cells through synergistic effect. The effect is significant. This method of combined treatment to improve migration and proliferation of vascular endothelial cells is worthy to be clinically applied and popularized. This may help patients with severe lower extremity ischemic disease in clinical practice, especially those who are unable to reconstruct blood vessels. However, the biosafety of galectin-3 or low molecular heparin has not been published in clinical therapy, particularly in the endothelial cell induced from bone marrow MSCs, so we need to explore more in the future.

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Disclosure of conflict of interest

None.

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