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The association of low molecular heparin and galectin-3 on the cell migration and proliferation of vascular endothelial cell from mesenchymal stem cells.

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Abstract

Objective: To explore the effect of the association of low molecular heparin and Galectin-3 on the cell migration and proliferation of vascular endothelial cell from mesenchymal stem cells.
Methods: Depending on the administration, this study is divided into four groups: low molecular weight heparin group, adding 20 μg/ml low molecular weight heparin into the cells; Galectin-3 group, adding 5 μg/ml Galectin-3 into the cells; combination group, adding 20 μg/ml low molecular weight heparin and 5 μg/ml of Galectin-3 into the cells; control group, equal volume of phosphate buffer saline buffer into the cells. Then we explored the effect of the association of low molecular heparin and Galectin-3 on the cell migration and proliferation of vascular endothelial cell from mesenchymal stem cells.
Results: The optical density at 490 nm (OD_{490}) for LMWH, Galectin-3, combined and control groups were 0.285 ± 0.018, 0.297 ± 0.041, 0.351 ± 0.016, and 0.233 ± 0.005, respectively, and the combined group could significantly increase the cell proliferation than another group (P<0.05). Cultured for 24 h, the cell migration rate of low molecular weight heparin group and Galectin-3 group were 42.02 ± 7.62 and 45.82 ± 3.96, respectively, whereas the cell migration rate of combined group and control group were 68.53 ± 11.22 and 34.21 ± 3.99, respectively, suggesting that combined group had the largest cell migration (P<0.05).
Conclusion: The association of low molecular heparin and Galectin-3 could significantly improve the cell migration and proliferation of vascular endothelial cell from mesenchymal stem cells.

Keywords: Low molecular heparin, Galectin-3, Vascular endothelial cell, Cell migration, Cell proliferation.

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Introduction

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 quality of life [1-3]. Among various types of PAD, arterial occlusive disease of low extremity and diabetic foot are the most serious, which are difficult to cure [4,5]. Recently years, PAD have aroused widespread attention due to it can lead to limb ischemia, necrosis, eventually amputation, or even death in patients [6,7]. It is reported that stem cell transplantation can be applied to treat PAD [8]. Similarly, it has been proved that transplanted bone marrow mesenchymal stem cells (MSCs) with great regenerative potential may differentiate into vascular endothelial cells and smooth muscle cells, and then to repair the damaged tissue [9]. At same time, through autocrine and paracrine pathway, it synthesizes and secretes vascular growth factor to promote angiogenesis. Another study shows that bone marrow MSCs can promote cell proliferation, suppress apoptosis, and through anti-inflammatory to promote angiogenesis [10,11]. Thus, vascular endothelial cell is the key factor in stem cell transplantation. Vascular endothelial cells that constitute the blood vessel wall act as a shield for harmful stimulus to blood. It is the sole anti-thrombotic cell type in human body [12-14]. In addition, it can produce multiple active substances to protect blood vessel [15-17]. However, clinical investigation found that some problems remain unsolved. MSCs-derived vascular endothelial cells play an important role on angiogenesis and repair of damaged vascular tissue, but so far, the migration and proliferation of MSCs-derived vascular endothelial cells may mainly influence the ability of angiogenesis and vascular repair. Therefore, how to improve the migration and proliferation of MSCs-derived vascular endothelial cells is the focus of clinical research for treating PAD.

Heparin is commonly used as anticoagulant drugs clinically to prevent postoperative thrombosis [17-20]. Low molecular weight heparin (about 5 kd) is generated through hydrolysis of heparin mainly. It has been widely used in clinical practice because it has many advantages, such as high efficiency, ineligible affinity to platelet and better stability. Galectin-3 belongs to glycoprotein. Depending on its glycol-domain, it specifically binds intracellular glycoproteins, cell surface molecules, glycosylated extracellular matrix proteins and membrane proteins *via* the lectin-glyco-interaction to participate in a variety of physiological and pathological

processes, including cell growth, apoptosis, cell adhesion, vascularization, tumour invasion, and metastasis [21-23].

Previous studies show that Galectin-3 may be involved in vascular endothelial cell migration, chemotaxis, and tumour angiogenesis in endothelial cells [24]. Therefore, this study is of great clinical value in the study of 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.

Materials and Methods

Experimental materials

The experimental animals were 20 healthy male specific pathogens free (SPF) Sprague Dawley (SD) rats, weighting 130-160 g. They were housed with a 12 h dark/light cycle with temperature 25°C and humidity (55%). Those rats were provided by animal experimental center of Anhui medical university, and the study was approved by the animal ethics committee of Anhui medical university with a permission number SCXK (Wan) 2005-001.

Cell Culture

Isolation and culture of MSCs

The rats were executed in sterile condition. After rats received the euthanasia, the shin and fibula of rats were separated under sterile condition. The marrow fluids were obtained by washing the marrow cavity with 5 ml low glucose-Dulbecco's Modified Eagle's Medium (L-DMEM). The cells were collected from marrow fluids through centrifugation at 1000 rpm for 5min. After phosphate buffer saline (PBS) was added in the acquired cells, a same value of 1.073 g/L^{-1} percoll was slowly added. Then, cells were centrifuged at 2400 rpm for 20 min, and milky white cells in interface layer were acquired. They were washed three times by PBS and inoculated in the 100-mm petri dish to culture with L-DMEM culture medium. The cells were cultivated at 5% CO_2 with a temperature of 37°C. The non-adherent cells were discarded after 48 h by washing the seeded cells with PBS and changing the medium. Then the cells were continuing cultured under the same conditions, and the medium was changed every 3 days. When cell confluence reaches 80%, the supernatant was discarded, and the cells were washed with PBS repeatedly, added with trypsin. After 2 to 3 min, the reaction was terminated by adding culture medium. The cells were beat repeatedly, centrifuged at 1000 rpm for 5 min, discarding the supernatant. Then the cells were inoculated in L-DMEM culture.

Subculture of cells

For 12 to 14d, the cell confluence reaches 80%, discarding the culture medium, washing with PBS 3 times, adding 0.125% trypsin to digestion for 2-3 min. The cells were observed under fluorescence microscope. When the structural cells became irregular and gap junctional intercellular enlarged, L-DMEM medium was added to terminate the digestion. The cells were passaged at a ratio of 1:2, by trituration when they reached 80% confluence.

Induction differentiation of MSCs

The cells of passage 4 were selected to test, which characters were stable. 10 ng/ml endothelial growth factor (vascular endothelial growth factor, VEGF) and 2 ng/ml alkaline fibroblast growth factor were added to cells, basic fibroblast growth factor, bFGF), co-cultured. The morphologic change of cells was constantly observed under Light microscope.

Immunofluorescence microscopy

The expression of vWF was observed using immunofluorescence staining. The passage 2 cells of experimental and control groups were plated in 6-pore plates with built-in slides. Primary antibody (rabbit anti-rat von willebrand factor (vWF), 1:40) was added, and the cells were incubated at 4°C overnight. After washing with PBS, a 1:10 dilution of fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG was used as a secondary antibody. Then, PBS washed, mount with glycerol, immediately observed under the fluorescence microscope.

Transmission electron microscopy of ultrathin sections

After the cells induced to differentiation for 14 days, they were washed with PBS. Then cells were transferred to 15 ml plastic centrifuge tube, and centrifuged at normal temperature. The cells were precipitated with 4% paraformaldehyde-2.5% glutaraldehyde, and fixed at 4°C for 2 h. Following the cells were dehydrated with gradient ethanol, semi thin sections were prepared, which stained with azure-methylene blue, then located under light microscope. Then ultrathin slices were mounted and counterstained with uranyl acetate-lead citrate, and observed under transmission electron microscope.

Vascular endothelial cell proliferation and migration

The proliferation activity of vascular endothelial cell was detected using 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) assay. The logarithmic growth phase cells were selected and plated in 96-well plates, each pore cell number of each pore is 1×10^5/L. After 24 h, all cells were adherent to the wall, completely replaced the DMEM medium without serum and continue to culture for 12 h. Experiment is divided into four groups: low molecular heparin group, added with low molecular heparin to final concentration of 20 μg/ml; Galectin 3 groups, added with Galectin-3 to final concentration of 5 μg/ml; Joint group: added with low molecular heparin to final concentration of 20 μg/ml and 5 μg/ml Galectin 3; Control group: added with the same volume of PBS buffer. All groups were cultured for another 24 h. Each pore was added with 150 μL dimethyl sulfoxide, shaking in shaking table at low speed for 10 min to make the crystal dissolves completely. The optical density at 490 nm (OD_{490})

for each well cells was measured. Within the scope of a certain number of cells, the amount determined by MTT crystallization is proportional to the number of cells. According to the measured OD value, the relative number of living cells and vitality were determined, the larger the OD value, the stronger the cell activity, and the bigger the quantity. The experiment repeated three times.

Flow cytometry detecting vascular endothelial cell cycles

Cell culturing and grouping method were as shown in the above. The cells were continue cultivated for 24 h, digested after collection, and washed with PBS repeatedly, adjusted cell for a total of 2×10^6/L. The cells were centrifuged at 1000 rpm for 5 min and washed with PBS. Away from the light, 1 ml of DNA dyeing was added to the cells, repeatedly beat and blended. 15 min later, the endothelial cell cycles were tested and calculated with flow cytometry.

Endothelial cell migration analysis: wound healing assay

Cell culture and the grouping situation were shown 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, discarding no-sticking cells, exfoliate cell, real-time shooting the dynamic situation of vascular endothelial cell migration for 24 h. Continuously shoot wound location, analyses the healing situation.

Statistical analysis

χ^2 test was used as count data, and analysis of variance was used as measurement data. The data was expressed as a mean ± standard deviation, and the SPSS20.0 software was applied to conduct the statistical analysis. $P<0.05$ was considered statistically significant.

Results

Subculture of MSCs

The subculture cells grow fast, adhered completely within 24 h, and integrated completely within 4-5 days. The cells showed a uniform long spindle, showed a neatly brush when grow in group, and showed a spiral pattern in vigorous growth period (Figure 1).

Differentiation of the mesenchymal stem cells to vascular endothelial cells

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

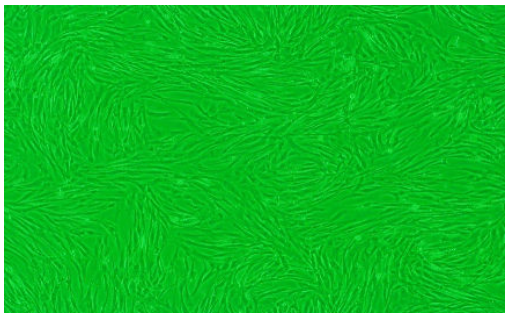

Figure 1. Demonstration of the morphology of the third generation of subcultured MSCs that were bone marrow-derived (40X).

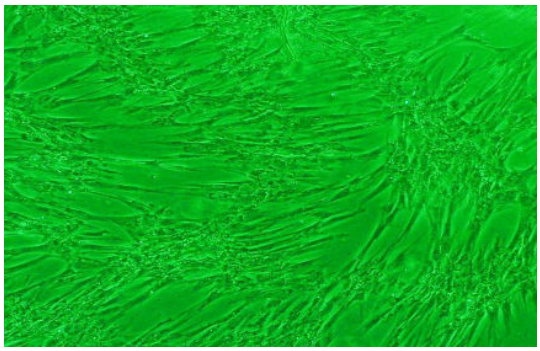

Figure 2. Observation of the morphology of MSCs that were differentiated into vascular endothelial cells by cocultures with 10 ng/ml endothelial growth factor, 2 ng/ml alkaline fibroblast growth factor and 2 ng/ml basic fibroblast growth factor. Cocultures were maintained for 20 d (100X).

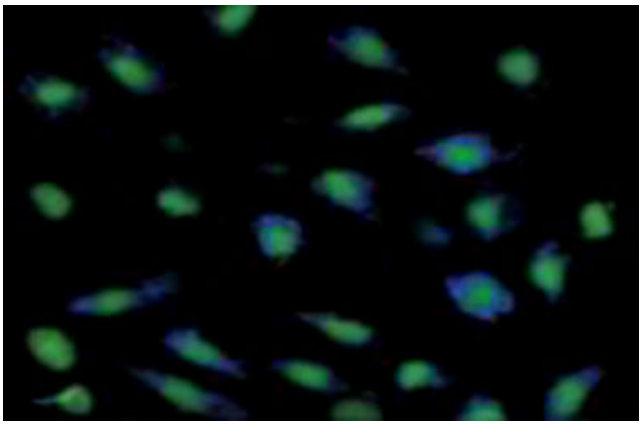

Figure 3. The immunofluorescence staining of MSCs differentiated into vascular endothelial cells that were positive for rabbit anti-rat von willebrand factor (vWF) (200X).

Immunofluorescence staining and electron microscope observation of induction cells

Immunofluorescence staining results: The immunofluorescence staining of differentiation of MSCs to endothelial-like cells presented a positive of vWF, and the cytoplasm of cells was present yellowish green and clear contour (Figure 3). The immunofluorescence staining of

undifferentiated MSCs cells showed no specific staining for vWF.

Electron microscope results: The cytoplasm was abundant in the induction cells, with more Mitochondria and Golgi complexes and pinocytosis vesicles, and with rougher endoplasmic reticulum and ribosomes scattered in. The typical Weibel-Palade (W-P) was seen in part of the differentiated MSCs cells (Figure 4).

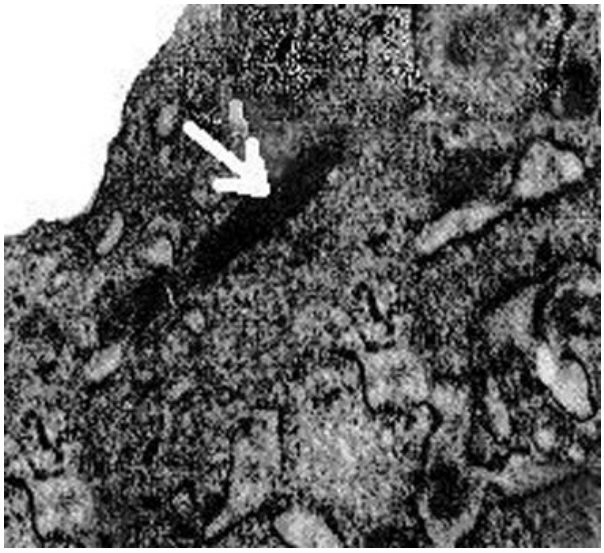

Figure 4. Electron microscopy was used to observe the cytoplasmic w-p bodies of MSCs cells at 14 days after induced differentiation.

The effects of each sample on OD490 value of vascular endothelial cells

Within the scope of a certain number of cells, the amount determined by MTT crystallization was proportional to the number of cells. The relative number and vitality of living cells were determined by the measured OD value, the larger the OD value, the stronger the cell activity, cell, the bigger the quantity. First, for the changing number of endothelial cells under the effect of different sample, we recorded the OD_{490} value. Each experiment repeated three times. As shown in Table 1, the OD value was biggest in joint group, which shows that on the action of low molecular heparin combined with Galectin 3, number of vascular endothelial cell proliferation is most obvious (P<0.05). In addition, low molecular heparin and Galectin-3 also can improve endothelial cells separately (P<0.05) (Table 1).

Table 1. *The effects of each sample on absorbance (OD_{490}) of vascular endothelial cells.*

Groups	Experiment numbers	OD490
Low molecular heparin	3	0.285 ± 0.018[a]
Galectin-3 group	3	0.297 ± 0.041[a]
Joint group	3	0.351 ± 0.016[a,b,c]
Control group	3	0.233 ± 0.005

[a]comparing to control group, P<0.05; [b]comparing to Low molecular heparin group, P<0.05; [c]comparing to Galectin-3 group, P<0.05.

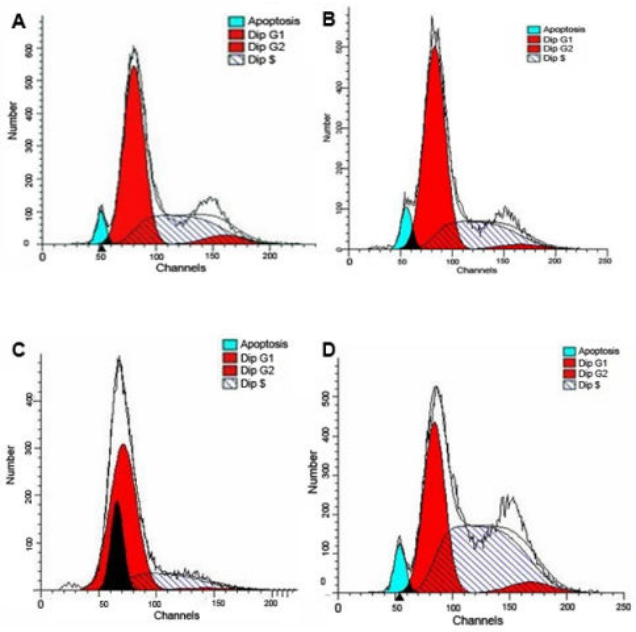

Figure 5. Differentiated endothelial cell cycle detected by flow cytometry, which were exposed to different concentrations for 24 h. A: Control group; B: Low molecular heparin group; C: Galectin-3 group; D: Joint group.

The effect of endothelial cell cycle changes of each group

The flow cytometry was used to analyse endothelial cell cycle changes. Each experiment was repeated three times, and we had recorded the different cycles. As shown in Table 2, the proportion of G0/G1 cells in the joint group was lowest, and the proportion of G0/G1 cells in low molecular heparin group with Galectin 3 was lower than that in control group (P<0.05). In addition, the percentage of cells in S phase was highest in the joint group, and the percentage of cells in S phase in low molecular heparin with Galectin 3 was higher than the control group (P<0.05). Joint group had the highest percentage of cells in G2/M phase, while low molecular heparin with Galectin group had higher G2/M phase cells than that of control group (P<0.05). The proliferation index of joint group was significantly higher than that of other groups, and the proliferation index of low molecular heparin with Galectin 3 group was higher than that of control group （P<0.05) (Figure 5 and Table 2).

Table 2. *The effect of each group on the changing of endothelial cell cycle ($\bar{x}$ ± sn=3).*

Groups	Experiment numbers	G0/G1 (%)	S (%)	G2/M (%)	PI (%)
Low molecular heparin	3	65.58 ± 1.65[a]	35.12 ± 0.97[a]	4.04 ± 0.83[a]	36.81 ± 1.45[a]

Galectin-3 group	3	67.32 ± 1.22[a]	33.76 ± 1.76[a]	3.92 ± 0.66[a]	34.66 ± 1.08[a]
Joint group	3	43.15 ± 2.65[a,b,c]	54.27 ± 1.43[a,b,c]	5.97 ± 0.38[a,b,c]	56.01 ± 0.78[a,b,c]
control	3	76.79 ± 2.77	29.53 ± 2.97	1.72 ± 0.46	22.85 ± 1.98

[a]comparing to control group, P<0.05; [b]comparing to Low molecular heparin group, P<0.05; [c]comparing to Galectin-3 group, P<0.05.

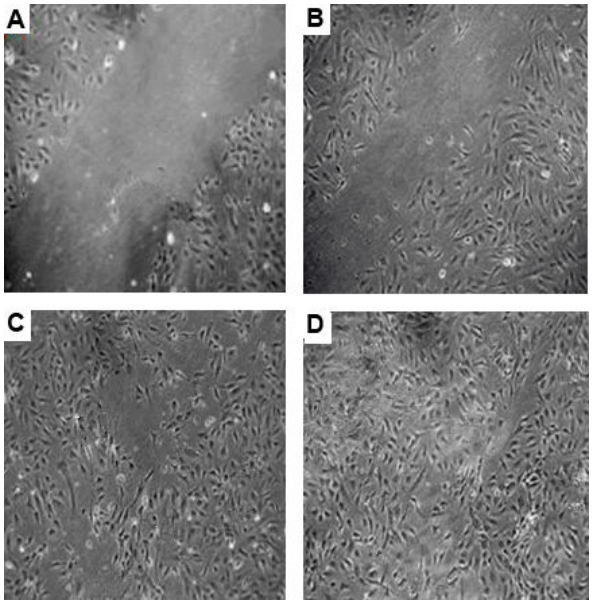

Figure 6. Effects of low molecular heparin combined with Galectin 3 on cell wound healing at different time points. A: observation the cell proliferation and migration distance at 0 h; B: observation the cell proliferation and migration distance at 6 h; C: observation the cell proliferation and migration distance at 12 h; D: observation the cell proliferation and migration distance at 24 h.

The effect of each group on the endothelial cell migration distance

Then we observed the number of vascular endothelial cells and cell migration distance under the effect of different samples, each experiment repeated three times. As shown in Table 3, the number of cells in joint group was biggest, and the maximum distance was longest, which showed that under the action of low molecular heparin with Galectin 3. The proliferation and cell migration distance of vascular endothelial cell significantly increased (P<0.05) at four different time point (0 h, 6 h, 12 h, and 24 h), and cell proliferation and migration ability were gradually enhanced during 0 h to 24 h. In addition, the low molecular heparin and Galectin-3 also can promote endothelial cell proliferation and cell migration distance separately (P<0.05) (Figure 6 and Table 3).

Table 3. *The effect of each group on the endothelial cell migration distance (x ± s, n=3).*

Groups	Cell number	Maximum distance (μm)
Low molecular heparin	28.83 ± 2.25[a]	402 .04 ± 62.52[a]
Galectin-3	32.98 ± 3.29[a]	399.18 ± 63.39[a]
Joint group	44.27 ± 5.29[a,b,c]	585.65 ± 44.52[a,b,c]
Control	12.32 ± 1.21	241.10 ± 53.26

[a]comparing to control group, P<0.05; [b]comparing to Low molecular heparin group, P<0.05; [c]comparing to Galectin-3 group, P<0.05.

Discussion

With the aging of society, the incidence of chronic peripheral arterial disease (PAD) increases year by year. And the trend is rising. 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 applied to treat PAD. However, clinical investigation found that some problems remain unsolved. The critical one is how to increase the migration and proliferation of vascular endothelial cells. The existing means are costive and inefficient. Therefore, how to improve the migration and proliferation of MSCs-derived vascular endothelial cells is the focus of clinical research.

This study selected low molecular heparin and Galectin 3 as the research object. The heparin is the commonly used anticoagulant drugs in clinical, widely used for postoperative prevention of thrombosis. Low molecular weight heparin (about 5 kd) is generated through hydrolysis of heparin mainly. It has been widely used in clinical practice because it has many advantages, such as high efficiency, ineligible affinity to platelet and better stability. Galectin-3 belongs to glycoprotein. Depending on its glycol-domain, it specifically binds intracellular glycoproteins, to participate in a variety of physiological and pathological processes.

First, this study analysed the vascular endothelial cell proliferation. Through the test for endothelial cell number, the results showed that low molecular heparin combined with Galectin 3 could significantly promote the proliferation of vascular endothelial cells (P<0.05). Compared to single low molecular heparin and Galectin 3, the proliferation effect significantly increased (P<0.05). It also showed the mechanisms of low molecular heparin and Galectin-3 to promote vascular endothelial cell proliferation was different. They had a collaborative relationship. Galectin 3 as a binding protein of IgE, can significantly stimulate the tube cavity structure of vascular endothelial cells, which generated from the bone marrow mesenchymal stem cells. Thus, more endothelial cells generate, so as to reach the proliferation of clinical effect. Low molecular heparin improves the endothelial cell proliferation by improving endothelial cell adhesion rate. Through two different mechanisms, low molecular heparin combined Galectin 3 effectively promote the vascular endothelial cell proliferation rate through synergy.

In conclusion, low molecular heparin combined Galectin 3 might significantly increase the migration and proliferation of vascular endothelial cells generated from bone marrow mesenchymal stem cells through synergistic effect. This study

may provide a theoretical basis for clinical popularization and application in future researches.

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