Basic Investigations

Effects of Panax Notoginseng Saponins on Homing of C-kit+ Bone Mesenchymal Stem Cells to the Infarction Heart in Rats

ZHANG Jin-sheng, HE Qing-yong, HUANG Tao, and ZHANG Bao-xia

Objective: To investigate the effects of panax notoginseng saponins (PNS) on homing of C-kit+ bone mesenchymal stem cells (BMSCs) to the infarction heart.

Methods: The acute myocardial infraction (AMI) model was established in 140 Wistar rats, 105 model rats survived after operation, and the model rats were randomly divided into five groups, 21 rats in each group: Western medicine group mobilized by subcutaneous injection of human granuloctye colony stimulating factor (G-CSF) 50 μg·kg-1·d-1; sham operation group and a model group treated by subcutaneous injection of normal saline 50 μg·kg-1·d-1; Chinese medicine group mobilized by intraperitoneal injection of Xuesaitong (血塞通) (ingredients of PNS) 150 mg·kg-1·d-1; integrative medicine group mobilized by subcutaneous injection of G-CSF 50 μg·kg-1·d-1 and intraperitoneal injection of Xuesaitong 150 mg·kg-1·d-1. Except for the sham-operated group, each group was divided into three sub-groups by three time points of 1 d, 7 d and 14 d. G-CSF was injected once a day for 7 d. Xuesaitong was injected once a day until the rats were killed. The flow cytometry was used for detection of C-kit+ cells in the peripheral blood in different time points, and immunohistochemical method was used for detection of the changes of C-kit+ cell and Ki-67+ cell numbers in the marginal zone of AMI.

Results: Twenty-four hours after the operation, C-kit+ cells had a slight increase in the model group compared with the sham operation group (P>0.05). The peripheral blood C-kit+ cells in the integrative group increased significantly compared with the other groups on 7 d and 14 d (all P<0.05). Meanwhile the expression of C-kit+ cells and Ki-67+ cells in the marginal zone of AMI in the integrative group increased significantly compared with the Chinese medicine group, the western medicine group and the model group on 1 d, 7 d and 14 d (all P<0.05), and the cells in the integrative group decreased significantly on 14 d compared with that on 7 d (P<0.05).

Conclusion: PNS can cooperate with G-CSF to mobilize C-kit+ BMSCs from the marrow into the peripheral blood and promote them “homing” to the infarction heart.

Keywords: Panax notoginseng saponins (PNS); bone mesenchymal stem cells (BMSCs); homing, experimental research

Autogenous bone marrow mesenchymal stem cells (BMSCs) can be mobilized after acute myocardial infraction (AMI), and home to the infarct site. In particular environments, BMSCs can differentiate into myocardial cells, vascular endothelial cells and smooth muscle cells, increasing functional myocardial cell number function, so as to improve the cardiac function. However, in normal circumstances, the content of peripheral blood stem cells is very low, far less than number and concentration needed for reparation of necrotic cardiocytes. Therefore, how to mobilize effectively a large number of BMSCs into the peripheral blood, and promote their homing to the target site to achieve the regeneration and restoration of myocardial function is a key to reduce the AMI mortality.

It has been reported that C-kit receptor, as the stem cell factor receptor protein in the cell membrane, is often referred to as CD117, which is an important cell surface antigen marker of BMSCs. The binding of stem cell factor and C-kit receptor can promote the proliferation and differentiation of hematopoietic stem cells and progenitor cells, which play an important role in the control of proliferation, differentiation and migration of stem cell, as well as the myocardial reparation. Ki-67 is a cell cycle specific antigen and expresses only in proliferation cells but not in stationary cells. It is a relatively definite indicator to determine cell proliferation. By use of detection of Ki-67 and C-kit receptor expression, it can be better studied how the outside interference factor to trigger the autologous BMSCs mobilization in AMI and to stimulate BMSCs “homing”
to the damaged heart, and how BMSCs participate in the mechanism of regeneration of cardiocyte and tissue repair under the micro-environment in heart.

MATERIALS AND METHODS

Drug Preparation
Xuesaitong Injection (血塞通注射液 composed of PNS, No.021016) was provided by Kunming Pharmaceutical Group Co., Ltd. Human granulocyte colony-stimulating factor (G-CSF) (Jin Lei Sai Qiang, No. 510980102) was provided by Changchun Jin-Sai Pharmaceutical Co., Ltd.

Reagents
RPMI 1640 medium was purchased from Shanghai Yu-Sen Biotechnology Co., Ltd. C-kit monoclonal antibody and Ki-67 monoclonal antibody kits were purchased from Beijing Bo-Ao-Sen Biotechnology Development Co., Ltd.

Instruments
Flow cytometry, was made by the FACS Calibur BD, USA; Animal breathing apparatus was produced by Shanghai Jiapeng Science and Technology Co., Ltd.; Multi-function ECG monitor was the product of Shanghai Photovoltaic Medical Electronic Apparatus Company.

Animals
One hundred and forty Wistar rats, 70 male rats and 70 female rats, aged twelve months, body weight (200±20) g, Grade II, and Certificate No. SCXK (Henan) 2005-0001, were provided by the Center of Animal Experiments, Henan Province and raised in the Center of Animal Experiments of Henan College of Traditional Chinese Medicine.

Establishment of Animal Model and Grouping
In reference to the literature,7 the animal model was established as follows: the rat was anesthetized with an intraperitoneal injection of 1% pentobarbital sodium (35 mg/kg), and was treated with tracheal intubation and connected to the small animal respirator for artificial breathing. Then the left anterior descending coronary artery was ligated after thoracotomy. The chest wall was sutured after electrocardiogram (ECG) showing AMI. Artificial inspiration was stopped when the physiological state of rat was steady. Penicillin was injected to prevent infection for 3 days. Eighteen rats died in modeling, 6 rats died within 6 h after modeling, 11 rats died 6-24 h after modeling.

Twenty-four hours after operation, 105 surviving rats were randomly divided into five groups: model group, Chinese medicine group, western medicine group, integrative group, sham operation group, 21 rats in each group. Except for the sham-operated group, each group was then divided into three subgroups by three time points of 1 d, 7 d and 14 d, and seven rats in each subgroup survived before they were killed at each time. For the sham operation group, the method was the same as the above except a loose knot was made after threading around the coronary artery.

Administration
The rat in the western medicine group was subcutaneously injected with G-CSF50 μg·kg⁻¹·d⁻¹, the sham operation group and the model group were subcutaneously injected with normal saline 50 μg·kg⁻¹·d⁻¹, the Chinese medicine group was mobilized by intraperitoneal injection of Xuesaitong (composed of PNS) 150 mg·kg⁻¹·d⁻¹, and the integrative group was mobilized by subcutaneous injection of G-CSF 50 μg·kg⁻¹·d⁻¹ and intraperitoneal injection of Xuesaitong 150 mg·kg⁻¹·d⁻¹. G-CSF was given once a day for 7 days. Xuesaitong was injected once a day until the rats were killed. Anesthesia with 1% sodium pentobarbital intraperitoneally (35 mg/kg), and 3 mL blood was collected from abdominal aorta.

Preparation of Samples
Seven rats in each group were anesthetized with 1% pentobarbital sodium (35 mg/kg) at 1, 7, 14 d after surgery, respectively. Blood of 3 mL was taken from abdominal aortic and heparin was used for anticoagulation. The rats were intracardially injected with 10% KCl solution 0.5 mL, with the heart stopped at diastole. Then the hearts were removed, rinsed by ice saline and fixed by formaldehyde. The left ventricle of heart was split into two parts along the long-axis mid-point, and the half of heart with cardiac apex was paraffin-embedded. The myocardium at the bottom of the half of heart with cardiac apex was continuously cut transversely into ten slices with thickness of 4 μm.

Flow Cytometric Analysis
C-kit⁺ BMSCs in peripheral blood at different time points were detected by flow cytometry. The cell density was adjusted to 100 μL mononuclear cell suspension containing 2 × 10⁶ cells with 10% FCS RPMI 1640 medium, and the supernatant was discarded following centrifugation. Then CD117-PE was added and fully shook. The cells were suspended by 1 mL washing liquid, and the supernatant was sucked and discarded following centrifugation. Afterwards, each sample was washed by 4 mL washing liquid with 1 × 10⁶ cells detected.

Immunohistochemical Analysis
Immunohistochemical method was used for detecting the changes of C-kit⁺ cell and Ki-67⁺ cell numbers in the marginal zone of AMI. Laboratory procedures were performed according to the kit manual (Boster Biological Engineering Co, Wuhan, China). Dyeing results was analyzed by the pathologic image analysis system, the average gray value for every 100 × field of vision in the marginal zone of AMI was calculated. 8 fields of vision were randomly chosen and determined in each slice, and
the average of them was used as the gray value. The cells of C-kit+ and Ki-67+ in 8 high power fields were observed under a 400× light microscope, and the number in each fields and the mean value were calculated.

Statistical Analysis
SPSS13.0 for Windows was used for the statistical analysis. All data were expressed as \( \bar{X} \pm s \) and compared by One-way analysis of variance and SNK-q. The difference before and after the treatment within groups were analyzed using paired t test. A value of \( P<0.05 \) was considered to be statistically significant.

RESULT
Comparison of C-kit+ BMSCs in Peripheral Blood at Different Time Points
The number of C-kit+ BMSCs in peripheral blood on 1 d, 7 d and 14 d did not change significantly in the sham operation group (\( P>0.05 \)). C-kit+ cells significantly increased in the Western medicine group and the integrative group but not in Chinese medicine group compared with the model group on 7 d and 14 d. It is suggested that the Western medicine has a faster effect of mobilization than Chinese medicine. While it did not show significant difference on 14 d and 1 d in the Western medicine group (\( P>0.05 \)), indicating that the rapid mobilization effect of Western medicine only lasted a short time. On the contrary, the content of C-kit+ cells continued to increase as compared between 14 d and 1 d in the Chinese medicine group, suggesting that the mobilization of BMSCs of the drug for invigorating blood circulation and eliminating stasis lasted longer. The number of C-kit+ BMSCs in peripheral blood in the integrative group increased significantly as compared with the Chinese medicine groups and the Western medicine group on 7 d and 14 d, suggesting that PNS may cooperate with G-CSF to mobilize C-kit+ BMSCs from marrow into the peripheral blood. (Table 1).

<table>
<thead>
<tr>
<th>Group</th>
<th>1 d</th>
<th>7 d</th>
<th>14 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation</td>
<td>0.13±0.14</td>
<td>0.14±0.02</td>
<td>0.13±0.02</td>
</tr>
<tr>
<td>Model</td>
<td>0.14±0.04</td>
<td>0.20±0.07</td>
<td>0.18±0.08</td>
</tr>
<tr>
<td>Chinese medicine</td>
<td>0.15±0.03</td>
<td>0.29±0.02</td>
<td>0.28±0.07</td>
</tr>
<tr>
<td>Western medicine</td>
<td>0.31±0.04</td>
<td>0.45±0.02</td>
<td>0.39±0.07</td>
</tr>
<tr>
<td>Integrative</td>
<td>0.37±0.02</td>
<td>0.54±0.07</td>
<td>0.48±0.05</td>
</tr>
</tbody>
</table>

Notes: Compared with the sham operation group, \(^* P<0.05\); Compared with the model group, \(^* P<0.05\); Compared with the integrative group, \(^* P<0.05\); Compared with 1 d in the same group, \(^* P<0.05\).

Comparison of C-kit+ BMSCs Homing to Marginal Zone of AMI at Different Time Points
There were no C-kit+ cells expressed in the sham operation group, while 24 h after the operation, C-kit+ cells could be occasionally observed in the marginal zone of AMI in the other groups, but being not obvious. Seven days later C-kit+ cells expressed highly in the integrative group, and relatively lower in the Western group, with a little C-kit+ cells seen in the model group. The number of C-kit+ cells decreased significantly on day 14 than on day 7 in all the groups, which is possibly caused by cell differentiation, leading to reduction of surface antigens of C-kit+ (Figure 1-13 and Table 2)

Effects of BMSCs on Ki-67+ Cells in Marginal Zone of AMI
There was no Ki-67+ cells found in the sham operation group, while 24 h after the operation, Ki-67+ cells could be occasionally observed in the marginal zone of AMI in the other groups, but being not obvious. Seven days later Ki-67+ cells expressed highly in the integrative group, and relatively lower in the Western group, with a little Ki-67+ cells in the model group. The number of Ki-67+ cells decreased obviously on day 14 than on day 7 in all the groups. (Figure 14-26 and Table 3).

Figure 1. The sham operation group (1 d, 7 d, 14 d), there was no C-kit+ cells with brown cytoplasm. × 400
Figure 2. The model group (1 d), there was no C-kit+ cells in and around the region of AMI. × 400
Figure 3. The model group (7 d), a lot of C-kit+ cells with brown cytoplasm can be seen in the edge of AMI district. × 400
Figure 4. The model group (14 d), there were less C-kit+ cells in the edge of AMI district, compared with in 7 d. × 400
Figure 5. The Chinese medicine group (1 d), there was no C-kit + cells. × 400

Figure 6. The Chinese medicine group (7 d), C-kit + cells with brown cytoplasm can be seen in the edge of AMI district, with little difference compared with model group in 7 d × 400

Figure 7. The Chinese medicine group (14 d), there were obviously less C-kit + cells with brown cytoplasm in the edge of AMI district, compared with that on 7 d. Some of these cells degenerated to cardiomyocyte-like cells. × 400

Figure 8. The western medicine group (1 d), there was no C-kit + cells. × 400

Figure 9. The western medicine group (7 d), there were obviously more C-kit + cells with brown cytoplasm in the edge of AMI district, compared with the Chinese medicine group in 7 d. × 400

Figure 10. The western medicine group (14 d), there were obviously less C-kit + cells with brown cytoplasm in the edge of AMI district, compared with that in 7 d. Some of these cells degenerated to cardiomyocyte-like cells. × 400

Table 2. Comparison of C-kit + BMSCs in rats marginal zone of AMI at different time points

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>1 d</th>
<th>7 d</th>
<th>14 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Model</td>
<td>21</td>
<td>2.6±1.4</td>
<td>3.7±0.9α</td>
<td>2.3±1.2α</td>
</tr>
<tr>
<td>Chinese medicine</td>
<td>21</td>
<td>3.3±1.3β</td>
<td>5.2±1.1∗β,△β</td>
<td>4.1±0.8∗β,△β</td>
</tr>
<tr>
<td>Western medicine</td>
<td>21</td>
<td>4.3±2.9</td>
<td>8.2±0.9∗β,△β</td>
<td>7.1±2.1∗β,△β</td>
</tr>
<tr>
<td>Integrative</td>
<td>21</td>
<td>5.1±1.4</td>
<td>15.2±2.3∗β,△β</td>
<td>10.2±1.9∗β,△β</td>
</tr>
</tbody>
</table>

Notes: Compared with the model group, *P<0.05; Compared with the integrative group, △P<0.05; Compared with 1 d in the same group, P<0.05; Compared with 7 d in the same group, αP<0.05.

Figure 11. The integrative group (1 d), there were a few C-kit + cells with brown membrane and cytoplasm. × 400

Figure 12. The integrative group (7 d), there were obviously more C-kit + cells with brown membrane and cytoplasm in the edge of AMI district, compared with western medicine group in 7 d. × 400

Figure 13. The integrative group (14 d), there were obviously less C-kit + cells with brown membrane and cytoplasm in the edge of AMI district, compared with that in 7 d. Some of these cells degenerated to cardiomyocyte-like cells. × 400

Figure 14. The sham operation group (1 d, 7 d, 14 d): × 400, there was no Ki-67+ cells.

Figure 15. The model group (1 d): × 400, there was no Ki-67+ cells.

Figure 16. The model group (7 d) × 400, Ki-67+ cells with leonine nucleus can be seen in the edge of AMI district.

Figure 17. The model group (14 d): × 400, there were less Ki-67+ cells in the edge of AMI district, compared with in 7 d.
Figure 18. The Chinese medicine group (1 d): × 400, there was no Ki-67+ cells.

Figure 19. The Chinese medicine group (7 d): × 400, Ki-67+ cells with leonine nucleus can be seen in the edge of AMI district, with little difference compared with model group in 7 d.

Figure 20. The Chinese medicine group (14 d): × 400, there were obviously less Ki-67+ cells with leonine nucleus in the edge of AMI district, compared with that in 7 d.

Figure 21. The Western medicine group (1 d): × 400, there were a few Ki-67+ cells.

Figure 22. The Western medicine group (7 d): × 400, there were obviously more Ki-67+ cells with leonine nucleus in the edge of AMI district, compared with Chinese medicine group in 7 d.

Figure 23. The Western medicine group (14 d): × 400, there were less Ki-67+ cells with leonine nucleus in the edge of AMI district, compared with that in 7 d.

Figure 24. The integrative group (1 d): × 400, there were a few Ki-67+ cells with leonine nucleus.

Figure 25. The integrative group (7 d): × 400, there were obviously more Ki-67+ cells with leonine nucleus in the edge of AMI district, compared with Western medicine group in 7 d.

Figure 26. The integrative group (14 d): × 400, there were obviously less Ki-67+ cells with leonine nucleus in the edge of AMI district, compared with that in 7 d.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Comparison of Ki-67+ Cells in rats marginal zone of AMI at different time points (X ± s, cell / high power filed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>n</td>
</tr>
<tr>
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<td>Model</td>
<td>21</td>
</tr>
<tr>
<td>Chinese medicine</td>
<td>21</td>
</tr>
<tr>
<td>Western medicine</td>
<td>21</td>
</tr>
<tr>
<td>Integrative</td>
<td>21</td>
</tr>
</tbody>
</table>

Notes: Compared with the model group, *P<0.05; Compared with the integrative group, **P<0.05; Compared with 1d in the same group, ^P<0.05; Compared with 7 d in the same group, _P<0.05.

**DISCUSSION**

Therapy of mobilization of BMSCs is based on the re-understanding of the theory that adult stem cells still have certain developmental potential and can differentiate to varieties of cells along with widespread transport phenomena, known as “stem cell niche”. The theory considers that the stem cells can maintain the integrity of the body morphology and the stability of the functions through self-renewal and differentiation in physiological or pathological conditions, either replacing or repairing the dead tissues.8-9 Orlic, et al.10 found that injection of G-CSF in rat model with AMI could mobilize the endothelial progenitor cells, which could migrate to the infarct area via blood circulation, differentiate into endothelial cells, increase capillary density and reduce the apoptosis of cells in the myocardial infarction border zone. Wu, et al.11 also mobilized autologous MSBCs by injecting G-CSF into the AMI rat model, and found regeneration of myocardial cells, as well as vascular endothelial cell, in the myocardial infarction regions. Seiler, et al.12 treated 10 cases of aged patients with serious unstable angina by injection of G-CSF and observed collateral circulation of heart in the treatment group, and found that the collateral circulation had improved 2 weeks later, suggesting the effectiveness of the mobilization of stem cells in short-term treatment.

Currently, there are many clinical and experimental studies about medicines for activating blood circulation.
and removing blood stasis. It has been proved that these medicines can improve the swelling, necrosis and apoptosis of vascular endothelial cells in the AMI ischemic region and the function of blood vessels, increase blood flow and reduce blood viscosity, and improve the microcirculation of AMI, and so on. However, the research about effects of the medicine for activating blood circulation and removing blood stasis on BMSCs mobilization and homing to infarction heart is still in its infancy, with a lot of questions to be further studied. For example, whether the medicine for activating blood circulation and removing blood stasis can mobilize BMSCs migrating, surviving, proliferating and differentiating to myocardial cells in the ischemic area? How they impact on angiogenesis? How they influence the heart function? To solve these problems is important for revealing the mechanism of the medicine for activating blood circulation and removing blood stasis in treatment of AMI.

The authors’ study showed that 24 h after the AMI, C-kit+ cells had a slight increase in the model group compared with the sham operation group (P>0.05), which suggested the mobilization and the proliferative potency of autologous stem cell were relatively weak. The peripheral blood C-kit+ cells in the integrative group increased significantly than that of the other groups on 7 d and 14 d, indicating Chinese medicine with G-CSF could increase the number of stem cells in peripheral blood and the effect continued for a long time. Meanwhile the expression of C-kit+ cells and Ki-67+ cells in the marginal zone of AMI in the integrative group increased significantly compared with the Chinese medicine groups and the western medicine group on 1 d, 7 d and 14 d (all P<0.05), indicating that the effect of western medicine combined with Chinese medicine was significantly better than that of Chinese medicine or Western medicine alone. The cells in the integrative group decreased significantly on 14 d compared with that on 7 d, suggesting that there was a peak time in the duration of homing of the stem cells. Therefore, PNS could cooperate with G-CSF to mobilize C-kit+ BMSCs from marrow into the peripheral blood, and promote them "homing" to the infarction heart.

REFERENCES


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