Effect of spleen-invigorating, Qi-replenishing and blood-arresting formula on zebrafish models with simvastatin-induced hemorrhage caused by spleen failing to control blood, in terms of theory of Traditional Chinese Medicine


Methods: In the first experiment, 60 AB strain wild-type zebrafishes were randomly assigned into two groups: normal group and model group. The model group was treated with 50 µM simvastatin for 24 h. The second experiment: The melanin allele mutated Albino strain zebrafishes were divided into normal, model, A group and B group. The observational parameters were as follows: blood flow, velocity of movement, hemorrhage ratio and improvement ratio of hemorrhage.

Results: Hemorrhage ratio: in the first experiment, brain hemorrhage ratio was 75%. In the second experiment, heart hemorrhage ratio was 65%. Blood flow: compared with the normal group, there was a significantly decrease in the model group (P < 0.001). Velocity of movement: in the first experimental, compared with the normal group, there was a significantly decrease in the model group (P < 0.001). Improvement ratio of hemorrhage: agents A had little effect in heart hemorrhage of the zebrafish; agents B could reduce heart hemorrhage ratio of the zebrafish, and increase the improvement ratio of hemorrhage.

Conclusion: The manifestation of zebrafish model with simvastatin-induced hemorrhage is basically similar to that of the clinical symptom pattern caused by spleen's failure to control blood. The Spleen-invigorating, Qi-replenishing and Blood-arresting Formula can reduce the heart hemorrhage ratio of zebrafish induced by simvastatin, and increase the improvement ratio of hemorrhage.

Keywords: Simvastatin; Hemorrhage; Spleen failure governing blood; Reinforcing Qi strengthening spleen; Reinforcing Qi arresting bleeding; Zebrafish
INTRODUCTION

Symptom pattern identification plays a significant role in the diagnosis and treatment of diseases in Traditional Chinese Medicine (TCM). The pattern identification is performed by using TCM four diagnostic methods. However, the integration of the symptom patterns with the diseases takes both of the patterns and characteristics of disease into consideration. In case of a simple disease, it can precisely reach a dual effect of the symptom pattern and disease. However, when it comes to some complicated or intractable diseases, it’s also valuable to think highly of symptom pattern and disease respectively. In basic, pharmacological, or toxicological research, we usually have to establish animal models that are integrated of symptom pattern and disease to research pathogenesis. Nevertheless, the animal models whose prognosis is intervened artificially can’t reflect the natural law of disease in human beings. We hold that since disease model can be established, it should be reasonable for finding symptom pattern through investigating the change of animals’ behavior and parameters. Based on TCM theory of ‘spleen governing limbs’ and ‘spleen governing blood’, zebrafish model with simvastatin-induced hemorrhage was established to investigate, effect of spleen-invigorating, Qi-replenishing herbs and spleen-invigorating, Qi-replenishing and blood-arresting formula.

MATERIALS AND METHODS

Laboratory animals

Species of zebrafishes: 60 AB strain wild type 1-day old zebrafishes after fertilization (1 dpf), which breed in natural pairs, were applied to the experiment. 360 melanin allele mutated Albino strain 3-days old zebrafishes after fertilization (3 dpf) were used.

Feeding condition: two kinds of zebrafishes were raised in water at 28 °C (water quality: 200 mg instant sea salt was added into every 1 L of reverse osmosis water with electrical conductivity 480-510 μs/cm; pH 6.9-7.2; water hardness: 53.7-71.6 mg/L CaCO₃). Animal permit number: SYXK (Zhe) 2012-0171.

Laboratory condition: The zebrafish facility at Hunter Biotechnology, Inc., was accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) international (Certificated No. 001458).

Reagents and apparatus

Hemorrhage-induced drug: Simvastatin, white powder, lot number: 404-520-2 purchased from TCI, Japan. The reserve liquid at a concentration of 50 mM was prepared by 100% DMSO, −20 °C saved stood by.

Methyl cellulose, white fiber, purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, MO, USA). Dissecting Microscope (SZX7, OLYMPUS, Ltd., Tokyo, Japan); Camera attached to the microscope (TK-C1481EC, JVC, Yokohama, Japan). Heart Blood Flow Analysis System (Zebralab 3.3, PB2084C, ViewPoint, Ltd., Lyon, France). Behavior analyzer (V3, ViewPoint, Ltd., Lyon, France). Precision Electric Balance (CP214, OHAUS, NJ, USA).

Formulas

Based on principle of blind design, formulas with different therapeutic principles were prepared by project leader. The experimental formulas were produced by Kangteng Pharmaceuticals, Inc., Beijing, according to the technology of water-extraction for granules. The granules were subsequently packed and labeled A and B, respectively. The packed granules were handed over to the experimenters in the research unit (Hunter Biotechnology, Inc.), who, under blind conditions, conducted statistical analysis of the experimental data. The statistical results were then transferred to the project leader and herb-preparation staff, based on the number of experimental herbs, who determined the groups. The compositions of two formulas are as followed:

Group A (Group Spleen-invigorating and Qi-replenishing, SPQR): Danshen (Radix Salviae Miltiorrhizae), Baizhu (Rhizoma Atractylodis Macrocephalae), Fuling (Poria), stir-frying with liquid adjuvant Gancao (Radix Glycyrrhizae).

Group B (Group Spleen-invigorating, Qi-replenishing and Blood-arresting, SPQRBA): Danshen (Radix Salviae Miltiorrhizae), Baizhu (Rhizoma Atractylodis Macrocephalae), Fuling (Poria), stir-frying with liquid adjuvant Gancao (Radix Glycyrrhizae), Huangqi (Radix Astragali Mongolici), Eijao (Colla Corii Asini), Qiancao (Radix Rubiae Cordifoliae).

Experiment grouping

In the first experiment, 60 AB strain wild zebrafishes were randomized into normal group and model group respectively. Both of the two groups were placed into 6-well plates, 30 in each well. Model group was treated with 50 μM simvastatin for 24 h. The second experiment: 360 melanin allele mutated Albino strain zebrafishes were randomized into 6-well plates, 30 in each group. First, the zebrafishes were treated with experimental herbs A and B with the concentration of 100, 250, 500, 750, 1000 μg/mL, respectively for 4 h. Then, zebrafishes was treated with 50 μM simvastatin for 30 min.

Testing index

Blood flow detection: After the first experiment, 10 zebrafishes were chosen randomly from each group. After the second experiment, 10 zebrafishes were chosen randomly to be photographed by Heart Blood Flow Analysis System and calculated blood flow. Velocity of movement testing: after the first experiment, 10 zebrafishes were chosen randomly from the
20 zebrafishes to record the velocity of movement by behavior analyzer. Hemorrhage and hemostasis assessment: after the second experiment, 20 zebrafishes chosen randomly from each group were photographed to count the number of those with heart hemorrhage and calculate the hemorrhage ratio (S) and improvement ratio of hemorrhage. Improvement ratio of hemorrhage (%) = [(model group - experimental group) / (model group-normal group)] × 100%.

Statistical methods
The data were analyzed using SPSS 19.0 (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY, USA). Experimental data is expressed as mean ± standard deviation (x ± s). Analysis of variance and Dunnett’s t-test were performed to test the differences between groups. It was considered statistically significant when P < 0.05.

RESULTS
Zebrafish hemorrhage models
In the first experiment, obvious brain hemorrhage could be seen in 15 out of 20 AB strain zebrafishes, with a hemorrhage ratio of 75%. In the second experiment, heart hemorrhage could be seen in 13 melanin allele mutated Albino strain zebrafishes out of 20, with a hemorrhage ratio of 65%. The optical micrographs of brain hemorrhage and heart hemorrhage in normal and model group are showed in Figure 1.

Blood flow detection
In the first experiment, a comparison of the blood flow in normal and model group was statistically significant (P < 0.01). In the second experiment, a comparison of the blood flow in normal and model group was statistically significant (P < 0.01) (Figure 2).

Figure 2 Comparison of the blood flow in normal and model group
A: comparison of the blood flow in first experiment; normal group: treated only with zebrafish-raising water; model group: treated with 50 µM simvastatin for 24 h. Data are presented as mean ± standard deviation (n = 10); significant differences compared with normal group were designated as *P < 0.01.

Velocity of movement testing
The results in first experiment showed that a comparison of the velocity of motion in the normal group and model group was statistically significant (P < 0.01) (Figure 3).

Figure 3 Comparison of velocity of motion and moving track in the experiment
A: comparison of velocity of motion; B: moving track of normal group; C: moving track of model group. Normal group: treated only with zebrafish-raising water; model group was treated with 50 µM simvastatin for 24 h. Data are presented as mean ± standard deviation (n = 11). Significant differences compared with normal group were designated as *P < 0.001.

Hemostasis effects of intervening agents
The hemostasis effects of intervening agents in group SIQR and group SIQRBA is showed in Tables 1, 2, Figures 4, 5. There were obvious heart hemorrhage in model group and different concentrations of group SIQR, which was more outstanding in the groups of 250 and 500 µg/mL. Heart hemorrhage happened in the concentration of 100, 250, and 500 µg/mL in both of the two groups. However, there was an obvious de-
crease of heart hemorrhage in groups of 750 and 1000 μg/mL. Comparison between hemorrhage ratio and improvement ratio of hemorrhage of group SIQR and group SIQRBA are showed in Figure 6.

It can be seen from Table 1 that there were obvious heart hemorrhage in model group and different concentrations of group SIQR, which was more outstanding in the groups of 250 and 500 μg/mL.

### Table 1 hemorrhage ratio and improvement ratio of hemorrhage of group SIQR (n = 20)

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (μg/mL)</th>
<th>Hemorrhage (tail)</th>
<th>Hemorrhage ratio (%)</th>
<th>Improvement ratio of hemorrhage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>-</td>
<td>13</td>
<td>65</td>
<td>-</td>
</tr>
<tr>
<td>SIQR</td>
<td>100</td>
<td>15</td>
<td>75</td>
<td>-15</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>17</td>
<td>85</td>
<td>-31</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>17</td>
<td>85</td>
<td>-31</td>
</tr>
<tr>
<td></td>
<td>750</td>
<td>16</td>
<td>80</td>
<td>-23</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>16</td>
<td>80</td>
<td>-23</td>
</tr>
</tbody>
</table>

Notes: the model group was treated only with 50 μM simvastatin for 30 min; the SIQR groups were given 100, 250, 500, 750, 1000 μg/mL in the different experimental groups respectively 4 h, and then treated with 50 μM simvastatin for 30 min; SPQR: group spleen-invigorating and Qi-replenishing; "-": the improvement ratio of hemorrhage is negative value, which indicates bleeding aggravation.

### Table 2 hemorrhage ratio and improvement ratio of hemorrhage of group SIQRBA (n = 20)

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (μg/mL)</th>
<th>Hemorrhage (tail)</th>
<th>Hemorrhage ratio (%)</th>
<th>Improvement ratio of hemorrhage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>-</td>
<td>13</td>
<td>65</td>
<td>-</td>
</tr>
<tr>
<td>SIQRBA</td>
<td>100</td>
<td>11</td>
<td>55</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>12</td>
<td>60</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>9</td>
<td>45</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>750</td>
<td>5</td>
<td>25</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>5</td>
<td>25</td>
<td>62</td>
</tr>
</tbody>
</table>

Notes: the model group was treated only with 50 μM simvastatin for 30 min; the SIQRBA groups were given 100, 250, 500, 750, 1000 μg/mL in the different experimental groups respectively 4 h, and then treated with 50 μM simvastatin for 30 min; SPQRBA: group spleen-invigorating, Qi-replenishing and blood-arresting. Heart hemorrhage happened in the concentration of 100, 250, and 500 μg/mL in both of the two groups. However, there was an obvious decrease of heart hemorrhage in groups of 750 and 1000 μg/mL.

### DISCUSSION

In order to explore the similarity between zebrafish hemorrhage model and symptom pattern of spleen’s failure to control blood, we selected hemorrhage, blood flow and velocity of movement as three basic conditions. The results of research Our findings are as followed: hemorrhage: In the first experiment, AB strain zebrafishes had brain hemorrhage after being

---

Figure 4 Hemostasis effects of intervening agents in SIQR groups
A-G: phenogram of heart hemorrhage in different concentrations group SIQR. A-G: normal, model, 100, 250, 500, 750, 1000 μg/mL. Normal group: treated only with zebrafish-raising water. The model group was treated only with 50 μM simvastatin for 30 min. The SIQR groups were given 100, 250, 500, 750, 1000 μg/mL in the different experimental groups respectively 4 h, and then treated with 50 μM simvastatin for 30 min; SPQR: Group spleen-invigorating and Qi-replenishing.
treated by simvastatin for 24 h. In the second experiment, the melanin allele mutated Albino strain zebrafishes had heart hemorrhage after being treated by simvastatin for 30 h. Simvastatin is a kind of statins, which is used for regulating the level of cholesterol and preventing of cardiovascular diseases. However, a long-term oral simvastatin can not only increase the risk of rhabdomyolysis, but also cause leucopenia, thrombocytopenia. It was also found that simvastatin has a function of antiangiogenesis. Previous studies showed that zebrafish had been established as an in vivo model to study hemorrhagic stroke and vascular integrity. Zebrafish brain hemorrhage by Statins induced has been used as a model of spontaneous intracranial hemorrhage working cerebrovascular protection at restoring vascular stability. As it was verified by the two experiments that simvastatin can induce zebrafish hemorrhage, and the results are consistent with

Figure 5 Hemostasis effects of intervening agents in SIQRBA groups
A: phenogram of heart hemorrhage in different concentrations group SIQRBA; A1-A7: normal, model, 100, 250, 500, 750, 1000 µg/mL; normal group: treated only with zebrafish-raising water; The model group was treated only with 50 µM simvastatin for 30 min; The SIQRBA groups were given 100, 250, 500, 750, 1000 µg/mL in the different experimental groups respectively 4 h, and then treated with 50 µM simvastatin for 30 min; SPQRBA: Group spleen-invigorating, Qi-replenishing and blood-arresting.

Figure 6 Hemostasis effects of intervening agents in group SIQR and SIQRBA
The SPQR and SIQRBA groups were given 100, 250, 500, 750, 1000 µg/mL in the different experimental groups respectively 4 h, and then treated with 50 µM simvastatin for 30 min; A: comparison of hemorrhage ratio between group SIQR and SIQRBA; B: comparison of improvement ratio of hemorrhage between group SIQR and SIQRBA. SPQR: Group spleen-invigorating and Qi-replenishing; SPQRBA: Group spleen-invigorating, Qi-replenishing and blood-arresting.
previous studies. Blood flow testing: in the two experiments, there was a significant difference of blood flow between normal group and model group, which showed that blood flow in normal group was faster than that in model group. Velocity of movement: the results of first experiment showed that velocity of movement in model group significantly decreased compared to that in normal group. Qi can promote the movement of blood, and spleen governs limbs. Deficiency of Qi can decrease the velocity of blood, and deficiency of spleen may cause weakness in limbs. Combined with the results of our research, it can be admitted that zebrafish model with simvastatin-induced hemorrhage has the basic characteristics of symptom pattern of spleen’s failure to control blood. 

To further verify that whether simvastatin-induced zebrafish hemorrhage model has the similar characteristics of clinical symptom pattern of spleen’s failure to control blood, from perspective of evidence-based medicine, we worked out the therapeutic principle of spleen-invigorating, and therapeutic principle of spleen-invigorating. Qi-replenishing and blood-arresting, according to TCM theory of spleen, Qi and blood. Besides, we tried to prove the zebrafish hemorrhage model has the basic characteristics of symptom pattern of failure of the spleen to control blood through herbs-intervening research. The results of our research are as followed: Different concentration groups of spleen-invigorating, Qi-replenishing formula, which is composed by Danshen (Radix Salviae Miltiorrhizae), Baizhu (Rhizoma Atractylodis Macrocephalae), Fuling (Poria), stir-frying with liquid adjuvant Gancao (Radix Glycyrrhizae), all had no effect for zebrafish heart hemorrhage. Different concentration groups of spleen-invigorating, Qi-replenishing and blood-arresting formula, which is composed by Danshen (Radix Salviae Miltiorrhizae), Baizhu (Rhizoma Atractylodis Macrocephalae), Fuling (Poria), stir-frying with liquid adjuvant Gancao (Radix Glycyrrhizae), Huangqi (Radix Astragali Mongolici), Ejiao (Colla Corii Asini), Qiancao (Radix Rubiae Cordifoliae), could decrease zebrafish heart hemorrhage ratio. The groups of 750 and 1000 µg/mL had the best effect. Above all, it could be carried out that: as is known to all, heart is the total power of blood circulation, in which Qi of heart plays the most important role. However, when Qi is sufficient but blood is deficient, heart will fail to promote and control the blood circulation together with spleen. This is why Spleen-invigorating, Qi-replenishing Formula alone cannot prevent or cure the pathological state of simvastatin-induced zebrafish hemorrhage. Theory of TCM advocates that Ejiao (Colla Corii Asini) is the product of flesh and blood, which has a function of nourishing Yin and tonifying blood. Nourishing Yin can make vessel smooth and moist. Tonifying blood can not only circulate together with Qi, but also has a function of hemostasis. Traditionally, Qiancao is used to treat different kinds of hemorrhage. Besides, its special function of removing blood stasis and dredging the meridian can make vessels unobstructed. As a result, though Qiancao acts just as a secondary role in the formula, it has its unique function and is the key role in spleen-invigorating, Qi-replenishing and blood-arresting. So it can be concluded that spleen-invigorating, Qi-replenishing and blood-arresting Formula is potent in improving zebrafish heart hemorrhage induced by simvastatin.

In order to further describe the similarity between zebrafish hemorrhage model and symptom pattern of spleen’s failure to control blood, we compared the characteristics of experimental pattern with that of the pattern in medical practice, so as to find the traits in common. According to TCM clinical theory, symptom pattern of spleen’s failure to control blood stands for many kinds of chronic bleeding caused by failure of the spleen to control blood. In fact, it is composed by the symptoms of chronic bleeding and deficiency of spleen, and can cause deficiency of blood. As a consequence, apart from chronic and repeated bleeding, there will also be lassitude of limbs, yellow or pale complexion, pale tongue with thin coating, and a thin pulse. These clinical traits are results of the application of TCM four diagnostic methods. Obviously, we could not get information of pattern that totally conformed to theory of TCM four diagnostic methods from mice model. However, strictly following the clinical pathogenesis, transforming the main symptoms by microcosmic means, or herbs intervening can help to get similar clinical traits. Consequently, we transformed lassitude of limbs into experimental animal’s velocity of movement, transformed Qi failing to promote blood circulation into blood flow of experimental animals, and applied herbs intervening to the experiment to assess the results. The experimental results are as followed: The hemorrhage of animal model, decreasing in velocity of movement, and reduction of blood flow are all highly relevant to clinical manifestation. Spleen-invigorating and blood-arresting herbs have an outstanding effect of hemostasis.

In conclusion, the characteristics of zebrafish model with simvastatin-induced hemorrhage are basically in accordance with that of the symptom pattern due to spleen failing to control blood. The model is a stable one for the study of hemorrhage with the symptom pattern of spleen’s failure to control blood.

REFERENCES


16  **Chen XX**. Research on test and extraction of effective ingredients in Ejiao. Ke Ji Zi Xun 2015; 13(2): 226.

