Investigation of hemostatic effect of Spleen-invigorating, Qi-replenishing and Blood-arresting Formula on simvastatin-induced zebrafish hemorrhage model

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KEYWORDS
Spleen-invigorating, Qi-replenishing and Blood-arresting Formula; AB strain wild type zebrafish; Hemostasis; Blood flow

Abstract
Objective: To observe the hemostatic effect of the Spleen-invigorating, Qi-replenishing and Blood-arresting Formula on simvastatin-induced zebrafish hemorrhage model compared with that of the Heat-clearing and Blood-cooling Formula.
Methods: AB strain wild type zebrafish were treated with 0.5 μM simvastatin for 24 hours to establish the zebrafish hemorrhage model. Under strictly blind experimental conditions, the zebrafish were then treated with experimental agents in different concentration within the maximum non-lethal dose. The intervention effect of the Spleen-invigorating, Qi-replenishing and Blood-arresting Formula was comprehensively assessed by examining the main observational parameters such as the bleeding reduction rate and hemostatic effect while referring to the additional parameters such as blood flow, improvement rate of blood flow, velocity of movement, improvement rate of motion under spleen-qi deficiency.
Results: Hemostatic effect: The bleeding rate and hemostatic rate in the zebrafish were respectively, 30%, 15%, 45%, 40% and 60%, 80%, 40%, 47% when equal concentration of 500 μg/mL, 1000 μg/mL were used. This showed that the experimental herbs B1 was superior to B2 in decreasing the bleeding rate and improving hemostatic effect in the zebrafish. Improvement of blood flow: With equal concentration, the experimental herb B1 was superior
Clinical observation revealed many disorders of the hematological system such as immunologic thrombocytopenic purpura (ITP), chronic aplastic anemia (AA), acute leukemia (AL) and myelodysplastic syndromes (MDS), etc. The manifestations of simvastatin-induced zebrafish hemorrhage are basically similar to that of failure of the spleen to control blood in human beings. The Spleen-invigorating, Qi-replenishing and Blood-arresting Formula has good hemostatic effect on simvastatin-induced hemorrhage in the zebrafish. It also boasts the functions of improving blood flow and velocity of motion in the hemorrhagic zebrafish. Such a conclusion provides the experimental basis for the treatment of the syndrome of failure of the spleen to control blood by this formula.

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**Introduction**

Clinical observation revealed many disorders of the hematological system such as immunologic thrombocytopenic purpura (ITP), chronic aplastic anemia (AA), acute leukemia (AL) and myelodysplastic syndromes (MDS), etc. that might cause hemorrhage. Their bleeding mechanisms are mainly associated with decreased peripheral blood platelet count and disturbed blood coagulation. Currently, in the treatment of such hemorrhagic conditions, approaches like platelet transfusion, drug hemostasis and adrenal cortex hormone are still commonly administered. But repeated use of these measures tends to lead to decreased efficacy because of the emergence of antibody of transfused platelet and hormone ineffectiveness and dependence. According to the theory of traditional Chinese medicine (TCM), acute bleeding cases are usually due to blood-heat while chronic cases due to failure of qi to control blood. It has been proved by clinical practice that above cases respond well to traditional Chinese therapies. But it is often combined with platelet transfusion, hemostatic drugs and hormones, making it fairly difficult to assess the hemostatic effect of its own. In order to objectively assess the hemostatic effect of traditional Chinese therapies, we have chosen the AB strain wild type zebrafish as the research subject to establish the simvastatin-induced zebrafish hemorrhage model. We have designed two TCM formulas: the first one consists of the herbs for invigorating the spleen, replenishing qi and arresting blood. The second one consists of herbs for clearing heat and cooling blood. After administration of the formula, we observed their effects on the blood flow and motion of the zebrafish so as to provide direct curative effect evidence for the first formula.

**Materials**

**Laboratory animal**

AB strain wild type zebrafish aged 1 day after fertilization (1 dpf) were raised in water at 28 °C (water quality: 200 mg/l 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). The zebrafish facility at Hunter Biotechnology, Inc. was accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

**Formulas and reagents**

Two formulas were adopted: (1) The Spleen-invigorating, Qi-replenishing and Blood-arresting Formula; (2) The Heat-clearing and Blood-cooling Formula (both in granule form). Two formulas were adopted: (1) The Spleen-invigorating, Qi-replenishing and Blood-arresting Formula; (2) The Heat-clearing and Blood-cooling Formula (both in granule form). In order to exclude the impacts of human factors on the experiment results, blind design was allied in the preparation of herbs, i.e. the formulas prescribed by the project leader based on the design brief were directly handed over to the manufacturer to produce the herbal granules. The granules were subsequently packed and labeled B1 and B2, respectively. The packed granules were handed over to the experimenters 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 who, based on the number of experimental herbs, determined that B1 was composed of the ingredients of the first formula: astragalus (Astragalus penduliflorus subsp. Mongholicus), root of hairy asiabell (Codonopsis pilosula (Franch.) Nannf.), tuckahoe (Poria cocos (Schw.) Wolf.), largehead atractylodes rhizome (Atractylodes macrocephala Koidz.), Colla Corii Asini, madder (Rubia cordifolia L.), honey-fried licorice root; B2 was composed of the ingredients of the second formula: buffalo horn, Rehmannia glutinosa Libosch, White Peony Root, Cortex Moutan. Twenty mg/mL mother liquid was prepared before the experiment. Hemorrhage-induced drug: Simvastatin, white powder, lot number: 404-520-2 purchased from TCI, Japan. The reserve liquid at a concentration of 0.5 mM was prepared by dissolving simvastatin in 100% DMSO. Fibrous methyl cellulose, purchased from Sigma, US.

**Experimental apparatus**

Dissecting Microscope (SZX7, OLYMPUS, Japan); Heart Blood Flow Analysis System (Zebralab3.3 PB2084C); 6-well plates (Nest Biotech); Behavioral Analyzer (V3, View Point Life Sciences).
Methods

Observation of the maximum herbs non-lethal concentration (MNLC)

All experimental procedures were performed by the Hunter Biotechnology, Inc. under blind condition. A total of 360 1 dpf AB strain wild type zebrafish were randomized into 6-well plates, 30 zebrafish in each well. The concentration of the experimental herbs B1 and B2 were set at 100 μg/mL, 250 μg/mL, 500 μg/mL, 1000 μg/mL, 1500 μg/mL, 2000 μg/mL, respectively. The zebrafish were treated with different concentration of herbs. The number of death in each group was calculated to determine the MNLC of the experimental herbs.

Assessment of the experimental herbs and hemostatic effect on the bleeding zebrafish

The dpf AB strain wild type zebrafish were randomized into 6-well plates, 30 in each well. Thirty zebrafish were assigned into the normal control group. The remaining 300 zebrafish were treated with 0.5 μM simvastatin for 24 hours so as to establish the bleeding model. After modeling, the 300 zebrafish were divided into the model group and experimental herbs intervention group. After the herbal granules at different concentration were given, the velocity of motion of 10 zebrafish chosen from each experimental group was recorded using the behavior analyzer. The improvement rates of motion were worked out using the following equation: improvement rate of motion (%) = [(experimental herb group − experimental herb group)/normal control group − normal control group)] × 100%.

Assessment of the effect of the experimental herbs on blood flow of the zebrafish

The dpf AB strain wild type zebrafish were randomized into 6-well plates, 30 in each well. Thirty zebrafish were assigned into the normal control group. The remaining 300 zebrafish were treated with 0.5 μM simvastatin for 24 hours so as to establish the bleeding model. After modeling, the 300 zebrafish were divided into the model group and experimental herbs intervention group. After the herbal granules at different concentration were given, 20 zebrafish chosen from each experimental group were photographed to count the number of those with hemorrhage and calculate the hemorrhage incidence (%). The improvement rates contributed by the intervention herbs were worked out using the following equation: improvement rates of zebrafish hemorrhage (%) = [(model control group − experimental herb group)/model control group − normal control group)] × 100%.

Impact of the experimental herbs on zebrafish movement

The dpf AB strain wild type zebrafish were randomized into 6-well plates, 30 in each well. Thirty zebrafish were assigned into the normal control group. The remaining 300 zebrafish were treated with 0.5 μM simvastatin for 24 hours so as to establish the hemorrhage model. After modeling, the 300 zebrafish were divided into the model group and experimental herbs intervention group. After the herbal granules at different concentration were given, the velocity of motion of 10 zebrafish chosen from each experimental group was recorded using the behavior analyzer. The improvement rates of motion were worked out using the following equation: improvement rate of motion (%) = [(experimental herb group − model control group)/normal control group − model control group)] × 100%.

Statistical methods

The experimental results were statistically analyzed by using SPSS 19.0. The results were again subject to variance analysis and Dunnett-t test. It was considered statistically significant when \( P < .05 \).

Results

Result of the MNLC of the experimental herbs

MNLC was explored based on the pre-designed concentration of the experimental herbs. The results are shown in Table 1.

It can be concluded from Table 1 that the MNLC of B1 is 1000 μg/mL and the MNLC of B2 is 1500 μg/mL.

Hemostatic effect on the hemorrhagic zebrafish

Hemorrhagic spot and bleeding patches were found mainly in the heads of the simvastatin-induced zebrafish (shown in Table 2). The results showed that hemorrhage were observed in 15 zebrafish out of 20 in the model group, with a bleeding rate of 75%. The hemostatic effect of the experimental herbs on the bleeding model is shown in Table 2 and Figs. 1–3.

It can be concluded from Table 2 that the experimental herb B1 and B2 prove to have therapeutic effect on the bleeding zebrafish, and the bleeding rate tends to decrease with the increased concentration.

Table 1: Experimental herb concentration—mortality data table (n = 30).

<table>
<thead>
<tr>
<th>Concentration (μg/mL)</th>
<th>Experimental herb B1</th>
<th>Experimental herb B2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mortality (n)</td>
<td>Mortality rate (%)</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>250</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1000</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1500</td>
<td>10</td>
<td>33.3</td>
</tr>
<tr>
<td>2000</td>
<td>30</td>
<td>100</td>
</tr>
</tbody>
</table>
patches in the simvastatin-induced zebrafish of the model group. This demonstrates that simvastatin can be chosen to duplicate the bleeding model used in the pharmacodynamics experiment.

Fig. 2 shows that at concentrations of 500 µg/mL and 1000 µg/mL, the bleeding rates are 30% and 15% in the B1 treated group, while those in the B2 treated group are 40% and 45%, respectively. The results indicate that B1 appears to be superior to B2 in reducing the bleeding rate. Fig. 3 shows that, at the concentration of 500 µg/mL and 1000 µg/mL, the hemostatic rates are 60% and 80% in the B1 treated group, while those in the B2 treated group are 40% and 47%, indicating that B1 might be superior to B2 in hemostatic effect.

**Table 2** The hemostatic effect of the experimental herbs on the bleeding model (n = 20).

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Experimental herb B1</th>
<th>Experimental herb B2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bleeding number (tail)</td>
<td>Bleeding rate (%)</td>
</tr>
<tr>
<td>100</td>
<td>14</td>
<td>70</td>
</tr>
<tr>
<td>250</td>
<td>14</td>
<td>70</td>
</tr>
<tr>
<td>500</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>1000</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>1500</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1** Phenogram of the bleeding zebrafish model.

**Figure 2** Bleeding rates with the use of experimental herb of B1 and B2.

**Figure 3** Hemostasis rate with the use of experimental herb of B1 and B2.

The experiment results showed that the blood flow in the control group and model group was respectively, 0.24 ± 0.02 (µL/s) and 0.10 ± 0.01 (µL/s). A comparison of the blood flow in these two groups was statistically significant (P < .001). This showed that there was decreased blood flow in the bleeding zebrafish and the establishment of model was successful; the effect of the experimental herb B1 and B2 on the bleeding volume is shown in Table 3 and Figs. 4–5.

It can be concluded from Table 3 that the experimental herb B1 and B2 prove to have significant improvement function in the blood flow of the bleeding zebrafish compared with the model group, in addition, the blood flow tends to increase when the concentration gets higher.

**Improvement of the blood flow in the hemorrhagic zebrafish**
The results of Figs. 4 and 5 show that the experimental herb B1 is superior to B2 in improving the blood flow and increasing the improvement rate of blood flow in the bleeding zebrafish when the equal concentration of the herbs are used.

**Improvement of the motion of the hemorrhagic zebrafish**

The experiment results showed that the velocity of motion in the model control group was $0.58 \pm 0.05$ (mm/s), and that in the normal control group was $1.74 \pm 0.09$ (mm/s). A comparison of the results in these two groups was statistically significant ($P < .001$) and also showed that the establishment of the model was successful. The moving track of the bleeding zebrafish is shown in Fig. 6; the effect of the experimental herb B1 and B2 on the velocity of motion in the bleeding zebrafish is shown in Table 4 and Figs. 6–8.

It can be concluded that, both experimental herb B1 and B2 prove to be conducive to the velocity of motion. The velocity of motion tends to increase with increasing concentration.

Fig. 6 shows that zebrafish in the normal control group could move quickly with conspicuous track while that in the model group hardly displays any moving track; with the increased concentration of the experimental herb B1 and B2, the moving track gradually becomes conspicuous.

The results in Figs. 7 and 8 show that with equal concentration, both experimental herb B1 and B2 prove to be able to increase the velocity of motion and promote the improvement rate of motion. Either in the concentration of 500 $\mu$g/mL or 1000 $\mu$g/mL, the experimental herb B1 proves to be superior to B2 in increasing the velocity of motion and promoting the improvement rate of motion.

**Discussion**

**Application of zebrafish to disease model**

As a common tropical fish, 3–4 cm in length, zebrafish have strong vitality. Zebrafish and human genes are homologous as highly as 87%, and its advantage as the model organism is very prominent. Therefore, zebrafish is an ideal model in the study of human disease, as well as its processes and treatment.7–9 Currently, the zebrafish model has been widely used in the study of cardiovascular and cerebrovascular diseases,10 mental illnesses,11 metabolic diseases,12 malignant tumor13 and anti-tumor angiogenesis,14 etc. There has also been remarkable achievement in the application of the zebrafish model in the study of rapid evaluation of drug selection, pharmacology and toxicology.15–17 Recent years, the use of the zebrafish model in the study of screening pharmacodynamics effects of traditional Chinese medicinal substances, pharmacology, toxicity evaluation, and drug metabolism has even been witnessed.18,19 All these fully demonstrate that zebrafish is already a widely accepted and used model organism in the field of disease research and drug screening.20

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**Table 3** The effect of the experimental herb B1 and B2 on the bleeding volume.

<table>
<thead>
<tr>
<th>Concentration ($\mu$g/mL)</th>
<th>Experimental herb B1</th>
<th>Experimental herb B2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood flow (µL/s)</td>
<td>Improvement rate (%)</td>
</tr>
<tr>
<td>100</td>
<td>0.15 ± 0.01*</td>
<td>36</td>
</tr>
<tr>
<td>250</td>
<td>0.15 ± 0.00**</td>
<td>38</td>
</tr>
<tr>
<td>500</td>
<td>0.19 ± 0.01***</td>
<td>61</td>
</tr>
<tr>
<td>1000</td>
<td>0.21 ± 0.01**</td>
<td>81</td>
</tr>
<tr>
<td>1500</td>
<td>0.21 ± 0.01**</td>
<td>81</td>
</tr>
</tbody>
</table>

Note: Compared with the model group, *$P < .05$, **$P < .01$, ***$P < .001$; compared with the experimental herb B2, $^\Delta P < .05$, $^\Delta^\Delta P < .01$, $^\Delta^\Delta^\Delta P < .001$. 

**Figure 4** Blood flow with the use of the experimental herb B1 and B2.

**Figure 5** Improvement rate of the blood flow with the use of the experimental herb B1 and B2.

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Application of the zebrafish to blood disease model

Zebrafish has already become the best biological model of hematopoietic regulation for its unique biological features and advantages. Progresses have been made in the use of the zebrafish model in the study of aplastic anemia and acute leukemia. Bleeding is considered to be a common symptom in the blood system disorders, which is often a potential risk factor for patient’s death. For acute bleeding symptoms, platelet transfusion and hemostatic measures usually achieve ideal outcomes, but there is no ideal treatment for chronic bleeding. According to the theory of TCM, chronic bleeding is caused by failure of the spleen to control blood. The application of the TCM principle of invigorating the spleen, replenishing qi and running blood to control bleeding has achieved promising outcomes.

Table 4  The effect of the experimental herb B1 and B2 on the velocity of motion.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Experimental herb B1</th>
<th>Experimental herb B2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Velocity of motion</td>
<td>Improvement rate (%)</td>
</tr>
<tr>
<td>100</td>
<td>0.79 ± 0.05</td>
<td>18</td>
</tr>
<tr>
<td>250</td>
<td>0.86 ± 0.05*</td>
<td>24</td>
</tr>
<tr>
<td>500</td>
<td>0.95 ± 0.07***</td>
<td>32</td>
</tr>
<tr>
<td>1000</td>
<td>1.23 ± 0.08***</td>
<td>56</td>
</tr>
<tr>
<td>1500</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Compared with the model control group, *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 6  Motion trajectory diagram of the hemorrhagic zebrafish.

Figure 7  The velocity of motion with the use of the experimental herb B1 and B2.

Figure 8  The improvement rate in motion with the use of the experimental herb B1 and B2.
arresting blood proved to produce satisfactory therapeutic effect. Because of the involvement of multiple factors in therapeutic evaluation, it is difficult to evaluate the effectiveness of Chinese herbs independently. But owing to the impact of many factors on treatment of diseases, it is usually not easy to independently assess the therapeutic effect of traditional Chinese medicine. The establishment of the rodent models for the study of the mechanism of TCM in the treatment of hemorrhagic disease could be a desirable option. However, the long duration of the research may introduce more interfering factors, thus the zebrafish hemorrhage model warrants further exploration into its advantages (directivity) in Chinese herbal hemostasis mechanism research. The drawbacks such as prolonged study periods and numerous variants still exist. Therefore, it deserves to carry out exploratory research on the hemostatic mechanism of medicinal herbs by using the bleeding zebrafish model characterized by the advantages of rapidness and perceptual intuition.

**Interpretation of the experimental results**

Strictly blind experimental results and statistical results are as follows: (1) Hemostatic effect: The zebrafish bleeding rate was 30% and 45%, 15% and 40%; hemostasis rate was 60% and 40%, 80% and 47% in the zebrafish treated with B1 and B2 at equal concentration of 500 μg/mL and 1000 μg/mL, respectively. B1 proves to be superior to B2 in reducing the bleeding rate and hemostatic effect. (2) Improvement of the blood flow: The experimental herb B1 is superior to B2 in improving the blood flow and increasing the improvement rate of blood flow when the equal concentration of the herbs is used. (3) Improvement of motion: With equal concentration, both experimental herb B1 and B2 prove to be able to increase the velocity of motion and promote the improvement rate of motion. Either with the concentration of 500 μg/mL or 1000 μg/mL, the experimental herb B1 proves to be superior to B2 in increasing the velocity of motion and promoting the improvement rate of motion.

Two additional indices were applied to test the superiority of B1 in hemostasis compared with B2. Blood flow and improvement rate of blood flow were selected based on TCM theory that spleen-qi deficiency leads to reduction in blood flow. Results indicated that B1 is superior in blood flow and improvement rate of blood flow in comparison with B2.

We have adopted two sets of additional indicators in order to further explain why the experimental herb B1 is superior to the experimental herb B2 in reducing bleeding. In light of the weakened blood circulation due to qi deficiency (spleen-qi deficiency), the first set of additional indicators is for assessing the blood flow and improvement rate of blood flow.

The result shows that the experimental herbs B1 is superior to B2 in improving the blood flow and increasing the improvement rate of blood flow when compared with the experimental herbs B2. Since fatigue is usually caused by qi deficiency, the second set of additional indicators is for evaluating the velocity of motion and improvement rate of motion. Once again, it is proved that either with the concentration of 500 μg/mL or 1000 μg/mL, the experimental herb B1 is superior to B2 in increasing the velocity of motion and promoting the improvement rate of motion.

The analysis and interpretation of the experimental results could arrive at the following conclusions: Simvastatin-induced zebrafish hemorrhage is characterized by superficial bleeding, decreased blood flow velocity and decreased velocity of motion, which are similar to the clinical manifestations of failure of the spleen to control blood; apart from its effectiveness in hemostasis, the first formula can also help to improve the blood flow and velocity of motion in zebrafish. Such a finding provides an objective basis for the use of the Spleen-invigorating, Qi-replenishing and Blood-arresting Formula in the treatment of chronic bleeding caused by failure of the spleen to control blood.

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**References**


