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RESEARCH ARTICLE

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

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Abstract

OBJECTIVE: To investigate the hemostasis effect of spleen-invigorating, *Qi*-replenishing and blood-arresting formula, on a zebrafish models with simvastatin-induced hemorrhage, and with symptom pattern caused by spleen failing to control blood, in terms of theory of Traditional Chinese Medicine (TCM).

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.[1] 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',[2] 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 ℃ (water quality: 200 mg instant sea salt was added into every 1 L of reverse osmosisi water with electrical conductivity 480-510 μs/cm; pH 6.9-7.2; water hardness: 53.7-71.6 mg/L $CaCO_3$). 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℃ 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 Kangren tang 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*), Ejiao (*Colla Corii Asini*), Qiancao (*Radix Rubiac Cordifoliae*). 20 mg/mL mother liquid was prepared by zebrafish-raising water before the experiment.

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 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