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The Establishment of Zebrafish Cultivation Method and the Effect of Traditional Chinese Herbal Medicine on its Growth

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Abstract

Objective

The aim of this study was to find an appropriate food for zebrafish larval cultivation and analyze the effects of *Polygala tenuifolia* Willd. (YZ), *Semen Ziziphi spinosae* (SZR) and fruits of *Alpinia oxyphylla* Miq (YZR) on the development of the embryonic nervous system.

Methods

60 well-developed 4 days postfertilization (dpf) larvae were randomly divided into group I and II (30 tails for each group). Group I was fed *Paramecium* and group II was fed dried brine shrimp powder. After a 15-day feeding period, the body length and survival rate between the two groups were compared. Then 100 zebrafish eggs were randomly divided into 5 groups, 20 eggs each, and cultured at 28ºC. The negative control group was cultivated in embryo culture medium, the positive control group was cultivated in 0.10 mg/mL folic acid solution, treatment groups were given 0.10 mg/mL water extract of YZ, 0.2 mg/mL water extract of SZR and 0.17 mg/mL water extract of YZR. The formation time and embryonic development of neural plate, nerve tube and brain were observed.

Results

The death rate larvae in group II at each developmental stage were significantly lower than those of group I ($p<0.05$), and the growth rate of body length in group II was significantly higher than that of group I. The survival rate of larvae in group II was significantly higher than that of group I ($p<0.05$) at the 15th day and a specific growth rate (SGR) of 3.20% was observed in group I and 7.25% SGR in group II. The formation times of neural plate, nerve tube and brain of YZR group were shorter than those of the YZ and SZR groups, and significantly different from those of the negative and positive control groups ($p<0.01$).

Conclusion

The survival rate and body length growth rate of larvae in group II were significantly higher than those of group I, which suggested that the dried brine shrimp powder could be used as an alternative food for the larvae. YZR treatment was shown to shorten the formation time of the embryonic nervous system, indicating that the water extract of YZR can influence the development of nervous system in the early embryonic stage.

Keywords: Zebrafish; Survival rate; Body length; Nervous system development

Introduction

Zebrafish (*Brachydanio rerio*) is a common tropical fish in India and Bangladesh. Zebrafish has high homology to human in the blood, visceral organs, visual system and central nervous system and other aspects, for up to 87%, and has, therefore, become another widely recognized vertebrate model animal for biological research [1,2]. Zebrafish are widely used in the field of drug development as a model animal because of its advantages such as small size, high throughput, low breeding cost, short spawning cycle and reliable experimental output [3]. With the increasing popularity of zebrafish in research, the successful breeding of zebrafish larvae becomes particularly important. The choice of food for larvae during the transition from endogenous nutrition to exogenous nutrition is of particular importance to the survival and growth of zebrafish [4]. In recent reports, although the Paramecium and Artemia as general food for zebrafish larvae, and the rotifers, yeasts, egg yolk granules and pellets are widely used as additional food among Chinese researchers (4-6). Studies have been performed on the characteristics of these diets, but overall, the survival rate of larvae remains relatively low. In this study, based on the previous reports and our experience, improvements of larvae diet were made to significantly increase the survival rate.

With the significant improvement in living standards and the growing awareness of health and disease prevention, drugs or supplements derived from traditional Chinese herbal medicine (TCM) are gaining popularity [7]. The establishment of the professional committee of the World Federation of Chinese Medicine Association of TCM and Supplements in December 2015 boosted the modernization and globalization of traditional Chinese medicine [8]. According to official statistics from the China Medicines and Supplements Import and Export Chamber of Commerce, the imports and exports of drugs and supplements in 2016 amounted to 103.4 billion U.S. dollars, an increase of 0.73% year-on-year. Medicinal and edible Chinese herb is an important part of the traditional Chinese medicine, and all 87 entries of the 2002 "List of Medicinal and Edible Chinese Herb" from the former Ministry of Health are traditional Chinese herbal medicine. In-depth study of Chinese herbal medicine not only reflects the traditional thought of " homology of food and medicine", but also caters to the recent advocacy to promote health through organic and natural food choices [9]. In this study, to understand the effects of several traditional Chinese herbal medicines on the growth and development of zebrafish, folic acid was selected as a positive control and the embryo culture medium as a negative control, with *Polygala tenuifolia* Willd. (YZ), *Semen Ziziphi spinosae* (Suanzaoren in Chinese, SZR) and *Alpinia oxyphylla* Miq (Yizhiren in Chinese, YZR) as medication administration groups. Among the three Chinese herbal medicines, SZR and YZR have been included in the list of medicinal and edible herbs. The dried seeds of *Zizyphus jujuba* Mill.var spinosa (Bunge) Hu ex H.F.Chou (SZR) has been reported to possess soothing effects to the nerves, as well as liver protecting, anti-oxidation and other effects [10], which can be used to treat neurasthenia, palpitation and emotional or mental disorders and other psychiatric diseases [11]. The dried ripe fruit of *Alpinia oxyphylla* Miq. (YZR) has been reported to protect nerves, improve learning and memory ability, and possess anti-oxidation, anti-aging, cardiac protecting and anti-stress effects [12,13], which is also a homolog of the medicinal and edible herbs commonly used in the clinical prevention and treatment of Alzheimer's disease [14]. The dried root of *Polygala tenuifolia* Willd. or *P. sibirica* L. (YZ) has been shown to possess soothing effect, anti-inflammation, anti-dementia, brain protection, antidepressant, anti-myocardial ischemia, and other pharmacological effects [15,16]. Folic acid (YS) is a water-soluble vitamin and is involved in many important reactions and the synthesis of critical metabolites in the body. Folic acid deficiency in human can lead to an increase in the incidence of fetal congenital heart and neural tube abnormalities [17], and similarly, folic acid deficiency in zebrafish can lead to embryos body axis abnormalities and axoplasmic developmental disorders [18]. Therefore, using folic acid as a positive control group of the growth and development of zebrafish in this study is suitable and informative. In addition, after inoculation and *in vitro* fertilization, the zebrafish can successfully produce hundreds of embryos. The *in vitro* embryo development at 1 day is equivalent to a 3-month embryonic development in human [19], and the embryos and larvae are optically transparent [20]. These advantages provide an outstanding theoretical basis and greatly improve the accuracy of observation upon *in vitro* and *in vivo* treatment [22].

Materials and Methods

Animal Experiment Ethics Statement

All animal studies were ethically reviewed and approved by the Committee of the Ethics on Animal Care and Experiments at The Research Center of Yunnan University of Traditional Chinese Medicine, and all the animal experiments were carried out in accordance with the approved guidelines.

Experimental Animals

Wild AB-type zebrafish and Paramecium were purchased from the Chinese Academy of Sciences Institute of Aquatic Life. Brine shrimp eggs were purchased from a local company (Aijia Pet Aquarium Supplies Co., Ltd.). The daily breeding of zebrafish is carried out in a type I 5-layer double-row breeding facility (purchased from Beijing Aisheng Technology Co., Ltd.). Zebrafish breeding and culture followed the Westerfield method [21] and brine shrimp culture followed the breeder's manual.

Reagents and Preparation

Preparation of embryo culture medium followed an established method (22), which was composed of 0.127 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 4.2 mM $NaHCO_3$ at pH 7.2. The extracts of *Polygala tenuifolia*, *Ziziphus jujuba,* and *Alpinia oxyphylla* were provided by Yunnan BaiYao Group Chemical Laboratory, with good water solubility after filtration, and the stock solution (10 mg/mL) was stored at 4°C. Folic acid (YS) (1 mg/mL) was purchased from China New Chemical Reagent Research Institute, Shanghai, and stored at 4°C in dark. Tricaine, used as an anesthetic, was purchased from Tianjin Wind Boat Chemical Reagent Technology Co., Ltd., and prepared in distilled water to 1 mg/mL and stored at 4°C [23].

Larvae Culture

Fertilized eggs were harvested and washed with pure water for 3 times. Then the fertilized eggs were transferred into the embryo culture medium and cultured in the BPH-9082 incubator. Well-developed larvae on day 4 were randomly divided into groups I and II, and each group was repeated 3 times. Each group was incubated in a 5 L glass beaker, equipped with a heating rod, thermometer and bubbling oxygen pump, with 24 h uninterrupted heating and oxygenation, to ensure the water temperature was between 26.5°C - 28.5°C and sufficient oxygen during the incubation period. The food was provided daily at 9:00 and 18:00 ad libitum. Group I was fed with *Paramecium* (density of ≥ 1000 per mL) and group II was fed with dried brine shrimp powder. The feeding and growth status of larvae in each beaker were observed daily, and the number of deaths was recorded. The mortality rate was compared using the chi-squared test. The body length (mm) of larvae was photographed and calculated using an Olympus-SZX16 inverted stereomicroscope at 0 d, 5 d, 10 d, and 15 d. The Specific Growth Rate (SGR) was calculated using the following formula: SGR = 100 (ln(Lt)-ln(L0)) / t, where L0 and Lt are the average body length (mm) of larvae at the beginning of the experiment (5 days of age) and at the end (20 days old), t is the number of days [5].

Preparation of Extracts

Polygala tenuifolia (YZ), *Ziziphus jujuba* (SZR) and *Alpinia oxyphylla* (YZR), 1 kg each, were extracted with 10 volumes of 70% ethanol 3 times for 1 h each extraction. The extracts were combined and concentrated to 1-2 L (to ensure no ethanol was left in the concentrate). The extracts were extracted with petroleum ether, ethyl acetate, and n-butanol, respectively. The extract was dried to obtain petroleum ether, ethyl acetate, and n-butanol fractions, and the remaining concentrate was dried to obtain the water extract. The extraction was carried out in the Chemical Research Department of Yunnan Baiyao Group, and YZ, SZR, and YZR water extracts were measured to be 112.38 g, 85.0 g, and 105.0 g, respectively.

Herb Treatment Administration

Based on the preliminary results, 100 of the normally developed follicle eggs were randomly divided into 5 groups, each repeated 3 times. The eggs were placed in one of the following media: 1) embryonic culture medium (Negative control), 2) 0.10 mg/mL folic acid (Positive control), 3) 0.10 mg/mL YZ, 4) 0.2 mg/mL SZR, and 5) 0.17 mg/mL YZR, and cultured at 28°C. The embryos were anesthetized with 1 mg/mL tricaine and placed under an inverted microscope to observe the nerve plate, neural tube and brain formation time and embryonic development and to determine whether the treatment(s) promoted the development of the central nervous system. Statistical analysis. All data were analyzed by one-way ANOVA and Tukey method using Excel and SPSS17.0 software ($p<0.05$). The experimental data were presented as mean ± standard error (mean ± SE).

Results

Effects of Food on Larvae Growth

As shown in Table 1, the mortality of group II was significantly worse than that of group I ($p<0.05$). The growth rate of body length was significantly higher in group II comparing to that of group I (Figure 1). On day 15, the SGR was 3.20% in group I and 7.25% in group II, and better average body length and survival rate were also observed in group II (Table 2).

Group	Day 0-5	Day 6-10	Day 10-15
I	31.11	16.67	5.55
II	12.22*	6.67*	1.11*
*p<0.05 comparing to Group I			

Table 1: Death rate (%) of zebrafish larvae at different development stages.

Group	Average body length /(mm)	Survival rate (%)
I	4.80±0.03	35.56
II	5.34±0.02*	80.00*
*p<0.05 comparing to Group I		

Table 2: Survival rate and average body length of two groups after 15 d.

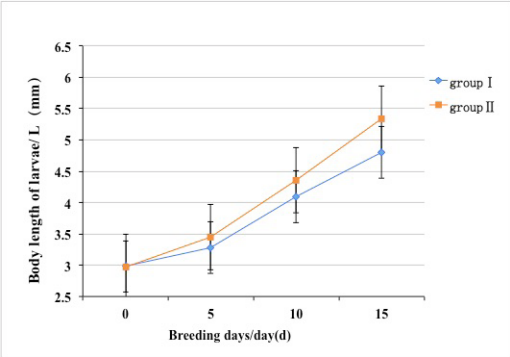

Figure 1: Larvae body length (day 0-15).

Effect of Herb Treatment on the Formation Time of Nervous System in the Larvae

After herb treatment, the formation times of the nerve plate, nerve tube, and brain were shortest in Positive control group, followed by YZR, Negative control group, SZR, with the YZ being the longest, which were significantly different from YS ($p<0.05$, $p<0.01$ and $p<0.01$, respectively) (Table 3-5; Figure: 2-4).

Group	Formation time (h)
Negative control	10.01±0.03*
YZ	10.10±0.07*
SZR	10.09±0.04*
YZR	9.97±0.04*
YS (Positive control)	9.87±0.07
*p<0.01 comparing to YS (Positive control)	

Table 3: Zebrafish neural plate formation time.

Group	Formation time (h)
Negative control	21.00±0.04*
YZ	21.25±0.06*
SZR	21.16±0.02*
YZR	20.93±0.06*
YS (Positive control)	20.83±0.02
*p<0.01 comparing to YS (Positive control)	

Table 4: Zebrafish neural tube formation time.

Group	Formation time (h)
Negative control	24.16±0.02*
YZ	24.50±0.02*
SZR	24.41±0.01*
YZR	24.05±0.02*
YS (Positive control)	20.83±0.02
*p<0.01 comparing to YS (Positive control)	

Table 5: Zebrafish brain formation time.

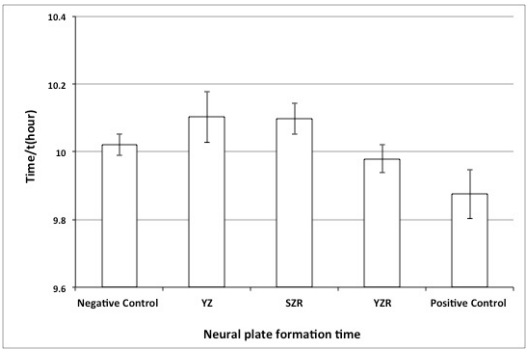

Figure 2: Effects of herb treatment on the formation of zebrafish neural plate.

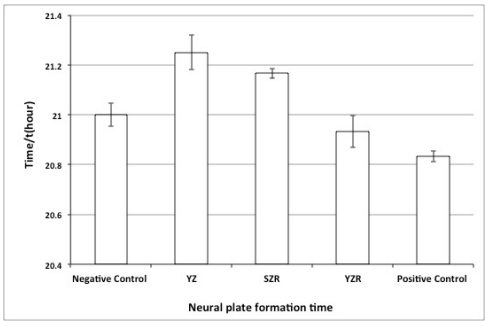

Figure 3: Effects of herb treatment on the formation of zebrafish neural tube.

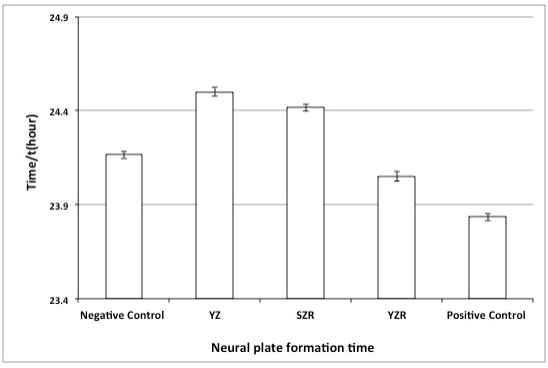

Figure 4: Effects of herb treatment on the formation of zebrafish brain.

Discussion

Polygala tenuifolia Willd. (YZ), *Semen Ziziphi spinosae* (SZR) and *Alpinia oxyphylla* Miq (YZR) have been widely used as herbal medicines in China for thousands of years. It was reported that the main component saponins isolated from YZ or SZR were found to have antipsychotic effects. In addition, pharmacological investigations have shown that the tepenes, diphenylheptanes and flavones were the main components in YRZ extract, which were found to have anti-inflammatory, anti-allergy and neuroprotective activities. The choice of food is an important factor for the survival

rate of zebrafish larvae. In the first 4 days of the development of the eggs, the hatched larvae receive nutritional support by absorbing the yolk sac [24]. Starting on the 5th day of development, most of the larvae begin to move and consume exogenous food. In addition to providing the necessary nutrients for the growth of larvae, a good food source shall be easy to prepare, store and clean up. *Paramecium*, as a common laboratory food for zebrafish larvae, is small in size and can scatter evenly, so that it can be easily consumed by the larvae. However, the cultivation of *Paramecium*, the replacement of culture medium, and passage can be costly in terms of time and effort, in practice, because *Paramecium* must be fed to larvae at a certain density. Based on previous experience, we found that, compared to *Paramecium*, dried brine shrimp powder not only greatly reduced the cost of production and storage but also performed well in feeding the larvae. Dried brine shrimp powder 1) can be easily processed to the required particle size for feeding the larvae, 2) has its own natural pigment, which helps to easily deduce the amount of food added and the amount of food consumed by the larvae. Residual food can be easily spotted and removed, which helps to reduce the pollution of water. In this study, we have also shown that the growth rate and survival rate of larvae fed with dried brine shrimp powder were significantly better than those of *Paramecium*. Therefore, dried brine shrimp powder can be a preferred food source for zebrafish larvae cultivation [25].

In the experiment of feeding the larvae, we only select the survival rate and the growth rate as a survey indicator under the consideration that larvae individuals are small and difficult to measure weight, so we didn't consider the weight gain rate that Shen Zhongming selected (4). The deficiency of our experiment is that the sedimentation rate, water resistance and decay time of two kinds of baits had not been measured and compared. In the water, fish excrement, bait and animal corpses and other organic decomposition of nitrogen will produce ammonia nitrogen decomposition. And non-ionic form of ammonia because of the absence of charge so that it can penetrate the cell membrane and show a toxic effect by the strong fat-soluble [26]. Zhou, et al. (27) used ammonia chloride to simulate ammonia nitrogen for toxicity test, and found the LC50 of ammonia nitrogen for 96h was 86.36mg / L, while Han Liqiang, et al. (28) found LC50 of 24,48,72,96h are 126, 114, 105, 101 mg /ml respectively. Therefore, when the laboratory decides to change the long-term bait with the input cost, the above indexes should be taken into account. In addition, in the choice of experimental fish of different diets, some researchers not only consider growth index like the visceral weight ratio and the relative fatness, but also consider physiological index such as the hemoglobin content, plasma Superoxide Dismutase (SOD) and erythrocyte SOD and so on, so we suggest that some indicators should be selected according to the needs of experiments during the process of young fish breeding.

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