Skin Toxicity of Wufang Babu Ointment to Zebrafish

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Abstract [**Objectives**] Using wild-type AB strain of zebrafish as experimental animal, this study investigated the damaging effect of Wufang Babu Ointment on skin cells, in order to evaluate the skin toxicity of Wufang Babu Ointment. [**Methods**] Wild-type AB strain of zebrafish with an age of 2 d were taken and fed in different concentrations of Wufang Babu Ointment solution for 24 h. The number of deaths in each group of zebrafish was recorded, and the mortality rate was calculated. Using Origin 8.0 software, the maximum non lethal concentration (MNLC) was simulated. Zebrafish raised in different concentrations of Wufang Babu Ointment solution for 24 h were placed under an anatomical microscope for taking photos, to analyze and calculate the incidence of skin damage in zebrafish. Based on the statistical analysis results of this indicator, the skin toxicity of Wufang Babu Ointment was evaluated. [**Results**] The MNLC of Wufang Babu Ointment on zebrafish was 671 μg/mL; Wufang Babu Ointment can induce skin damage at the concentrations of 224 μg/mL (1/3 MNLC) and 671 μg/mL (MNLC). [**Conclusions**] Wufang Babu Ointment had certain skin toxicity to zebrafish.

Key words Wufang Babu Ointment, Zebrafish, Skin toxicity

1 Introduction

Wufang Powder is a hospital preparation (Guiyaozhizi Z01060028) of the First Affiliated Hospital of Guangxi University of Chinese Medicine. It is mainly composed of Lycopi Herba, Rhei Radix Et Rhizoma, Eupolyphaga and other traditional Chinese medicines, and has the effects of promoting blood circulation, removing blood stasis, detumescence, relieving pain, and connecting bones and tendons[1-3]. Wufang Powder has obtained a large amount of clinical data, and is mainly used to treat osteoporosis, lumbar disc herniation, arthritis and other diseases[4-8], all of which have good clinical effects. The original powder has disadvantages such as rough preparation process, difficult quality control, quality difference, and low patient compliance. To overcome these shortcomings, the project team plans to develop it into babu ointment[9-10]. Babu ointment refers to a plaster made by mixing raw materials with suitable hydrophilic matrices and applying them onto backing materials. Its water-soluble matrix has good moisturizing properties, low skin irritation, and high bioavailability, which is suitable for a wide range of people. It has the characteristics of both ointment and hard ointment formulations, and a large drug loading capacity. It is particularly suitable for improving traditional Chinese medicine compound formulations with multiple components and large dosage[11-12], and has gradually become a substitute for traditional rubber patches and black ointments[13]. Wufang Babu Ointment restructured by the project team is a paste made by mixing Wufang Powder with suitable matrices such as kaolin and gelatin in a certain proportion, and then coating it on the backing material. The preliminary project team has conducted corresponding research on its formulation process and quality standards. Will the application of Wufang Babu Ointment have adverse effects on the skin after changing the dosage form? In order to explore this issue, zebrafish was taken as the research object, and the incidence of skin damage and cell apoptosis were explored, to evaluate the skin toxicity of Wufang Babu Ointment.

2 Materials and methods

2.1 Experimental animals Wild-type AB strain of zebrafish with an age of 2 d (2 dpf) were purchased from Hangzhou Huante Biotechnology Co., Ltd. License number for the use of experimental animals: SYXK (Zhe) 2022-0004. Zebrafish were raised in water at 28 ℃ for fish farming (electrical conductivity: 450 - 550 μS/cm; pH: 6.5 - 8.5; hardness: 50 - 100 mg/L $CaCO_3$), and breeding management met the requirements of international AAALAC certification (certification number: 001458). This experiment has been approved by the Experimental Animal Management and Use Committee of Hangzhou Huante Biotechnology Co., Ltd., and animal experiment ethics number: IACUC-2022-3309-01.

2.2 Drugs and reagents Acridine orange (AO) dye (lot number: C12894919, Shanghai Macklin Biochemical Technology Co., Ltd.); methyl cellulose (lot number: C2004046, Shanghai Aladdin Bio-chem Technology Co., Ltd.); DMSO (lot number: BCCD8942, Sinopharm Chemical Reagent Co., Ltd.); Wufang Babu Ointment (lot number: 20220314, provided by Formulation Center of the First Affiliated Hospital of Guangxi University of Chinese Medicine).

2.3 Main instruments Dissecting microscope (SZX7, Japan Olympus Optical Technology Company); CCD camera (VertA1, Shanghai Tusen Visual Technology Co., Ltd.); electric focusing continuous zoom fluorescence microscope (AZ100, Japan Nikon Company); precision electronic balance (CP214, USA Ohaus Company).

2.4 Preparation of drug The backing material was removed from Wufang Babu Ointment, and an appropriate amount of the ointment was taken and prepared with sterilized purified water into a solution of 1 g/mL, and it was prepared before use.

2.5 Determination of maximum non lethal concentration (MNLC)[14] The medicine solution of Section **2.4** was taken,

Received: January 20, 2024 Accepted: March 16, 2024

Supported by Guangxi Key R&D Plan (GUIKE AB20297010); Guangxi Science and Technology Base and Talent Special Project (GUIKE AD23023011); the National Natural Science Foundation of China (81960889).

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and an appropriate amount of sterilized purified water was added to dilute into a concentration of 0.6, 0.7, 0.8, 0.9, and 1.0 mg/mL for later use. 3 mL of each concentration of the medicine solution was taken into a 6-well plate, and 30 randomly selected 2 dpf wild-type AB strains of zebrafish were placed in each well. At the same time, a normal control was set up. 3 mL of sterilized purified water was added in the normal control, and 30 randomly selected 2 dpf wild-type AB strains of zebrafish were placed in the well. Under the same conditions, the number of deaths of zebrafish in each group was observed and recorded after 24 h of feeding at 28 ℃, and the mortality rate was calculated (Mortality rate = Number of deaths/Number of experiments × 100%). Using the concentration of Wufang Babu Ointment solution as the horizontal ordinate and mortality rate of zebrafish as the vertical axis, the maximum non lethal concentration was simulated using Origin 8.0 software.

2.6 Evaluation of skin damage in zebrafish[15] The medicine solution under Section **2.4** was taken, and an appropriate amount of sterilized purified water was added to dilute into a concentration of 74.6, 224.0, and 671.0 μg/mL for later use. 3 mL of each concentration of the medicine solution was taken into a 6-well plate, and 30 randomly selected 2 dpf wild-type AB strains of zebrafish were placed in each well. At the same time, a normal control was set up. 3 mL of sterilized purified water was added to the normal control, and 30 randomly selected 2 dpf wild-type AB strains of zebrafish were placed in the well. After 24 h of feeding at 28 ℃ under the same conditions, zebrafish were placed under an anatomical microscope to take photos, and the number of skin injuries of zebrafish at different concentrations was observed and recorded, and the incidence of skin injuries was calculated (Incidence rate of skin injury = Number of skin injuries/Number of experiments × 100%).

2.7 Statistical methods The maximum non lethal concentration was simulated using Origin 8.0 software. The evaluation results of skin injury were statistically analyzed using IBM SPSS 26.0 software, and pairwise comparisons between groups were performed using LSD-*t* test. $P<0.05$ showed significant difference, and $P<0.01$ showed extremely significant difference.

3 Results and analysis

3.1 Determination results of maximum non lethal concentration The number of deaths and mortality rate of zebrafish with different concentrations of Wufang Babu Ointment were shown in Table 1. The results showed that as the drug concentration increased, the number of zebrafish deaths also increased. According to simulation using Origin 8.0 software, the MNLC of skin toxicity of Wufang Babu Ointment on zebrafish was 671 μg/mL (Fig. 1).

3.2 Skin injury results The experimental results of skin damage after treatment with Wufang Babu Ointment were shown in Table 2, Fig. 2, and Fig. 3. The results showed that the incidence of skin damage in zebrafish in both the normal control group and the low-dose treatment group was 0. Compared with the normal control group, there was a significant difference in the incidence of skin damage of zebrafish in the medium-dose treatment group ($P<0.05$), and an extremely significant difference in the incidence of skin damage of zebrafish in high-dose treatment group ($P<0.01$). From this, it can be seen that Wufang Babu Ointment could induce skin damage at the concentrations of 224 μg/mL (1/3 MNLC) and 671 μg/mL (MNLC) under the conditions of this experiment.

Table 1 The number of deaths and mortality rate of zebrafish with different concentrations of Wufang Babu Ointment ($n=30$)

Group	Concentration μg/mL	Number of deaths	Mortality rate//%
Normal control	–	0	0
Wufang Babu Ointment	600	0	0
	700	2	7
	800	3	10
	900	16	53
	1 000	30	100

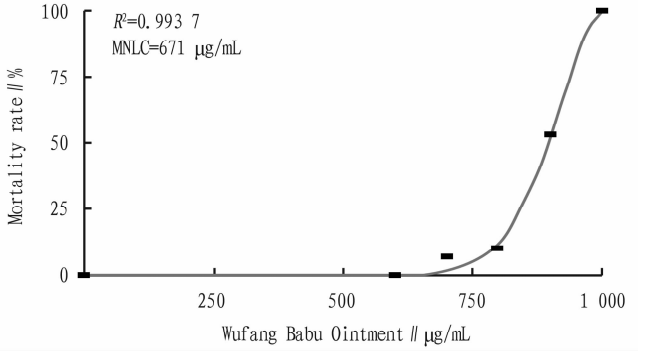
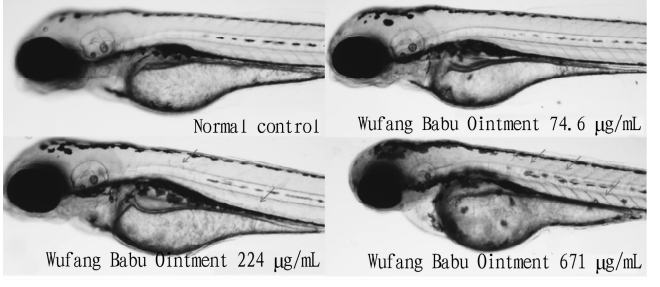


Fig. 1 " Concentration-death rate " curve of Wufang Babu Ointment

Table 2 Experimental results of skin damage after treatment with Wufang Babu Ointment ($n=30$)

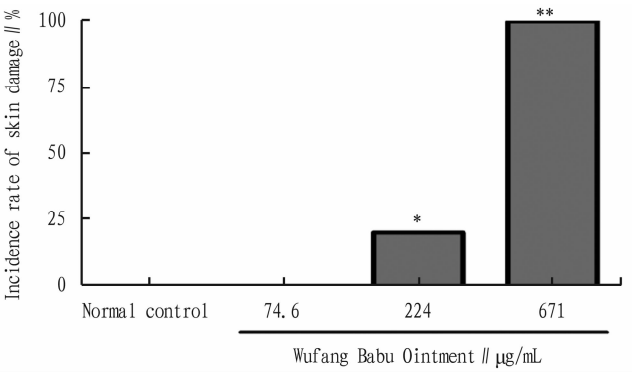
Group	Concentration μg/mL	Number of skin injuries	Incidence rate of skin injury//%
Normal control	–	0	0
Wufang Babu	74.6 (1/9 MNLC)	0	0
Ointment	224 (1/3 MNLC)	6*	20
	671 (MNLC)	30**	100

NOTE Compared with normal control, * showed $P<0.05$; ** showed $P<0.01$.



NOTE The arrow indicates skin injury.

Fig. 2 Typical image of zebrafish skin damage after treatment with Wufang Babu ointment



NOTE Compared with normal control, * showed $P<0.05$, and ** showed $P<0.01$.

Fig. 3 Incidence of skin damage in zebrafish treated with Wufang Babu Ointment

4 Discussion

Wufang Babu Ointment is a modified formula made from Wufang Powder and suitable matrix, and it contains the toxic traditional Chinese medicine Strychni Semen. The main component of Strychni Semen, strychnine, is both effective and toxic, and the therapeutic dose is very close to the toxic dose[16-17]. Therefore, there have been many reports of toxic side effects in its clinical application[18-21]. In order to ensure the medication safety of Wufang Babu Ointment, this experiment investigated the maximum non lethal concentration of Wufang Babu Ointment on zebrafish, and evaluated the skin toxicity of Wufang Babu Ointment based on the incidence of skin damage and zebrafish cell apoptosis. According to the above experiment, the maximum non lethal concentration of Wufang Babu Ointment on zebrafish was 671 μg/mL, and skin damage can be induced at a concentration of 224 μg/mL. It can be considered that Wufang Babu Ointment has certain toxicity to zebrafish skin. However, there is currently no obvious skin toxicity when used in clinical patients. Occasional symptoms such as itching in the external application area may occur, and it may have certain allergenicity due to personal physical differences.

In this paper, zebrafish was selected as the research object. Compared with other model organisms, zebrafish has its unique evaluation advantages. There are research reports indicating that zebrafish has a high degree of homology with the human genome, accounting for about 87%. It can reliably simulate and predict human physiology, presenting physiological characteristics similar to humans, and is a relatively mature animal model for evaluating drugs[22-26]. The skin structure of zebrafish is similar to that of human, and its skin gene expression is similar to that of human. Therefore, compared with other mammals, it has an unique advantage by using zebrafish to evaluate the skin toxicity of Wufang Babu Ointment[27]. Zebrafish has advantages such as small size, easy feeding, transparent embryos, and easy observation, making it a widely used model organism in medical and toxicological research. Due to its advantages of high-throughput screening, short testing cycle, and high reliability of experimental results, zebrafish has gradually been used for drug efficacy evaluation in recent years, and has become a recognized evaluation method in the pharmaceutical industry, which is also a hot research topic for scholars.

In practical applications, Park *et al.*[28-29] evaluated the whitening effect of cosmetics through image analysis. However, Cheng Li *et al.*[30] studied the anti-inflammatory activity of blood ginseng extract using zebrafish tail cutting method, and Wan Qingjia *et al.*[31] used LPS to induce oxidative stress in zebrafish embryos to evaluate the antioxidant effect of hemp leaf extract. Wan Qingjia *et al.*[32] also used 3 dpf zebrafish to evaluate the anti skin allergy effect of *Aloe vera* flowers. Therefore, it is reliable using zebrafish to evaluate skin toxicity in the experiment. In the experiment, the age of selected zebrafish was 2 d because the respiratory and esophageal tracts of zebrafish were closed at this time, and the absorption of oxygen and nutrient components was obtained through the surface skin. This can be used as a research model for the skin toxicity of topical drugs to some extent.

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