

# The influence of Yishen Ningxin formula on the behavioral and neurotransmitter changes in a zebrafish model of generalized anxiety

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**Abstract:** This study evaluated the anxiolytic effects of Yishen Ningxin formula (YSNX) in caffeine-induced zebrafish models and explored underlying mechanisms. Using 7-day post-fertilization AB zebrafish, anxiety-like models were established with 100μmol/L caffeine exposure. Groups included normal control, caffeine-only, and YSNX-treated cohorts (200/550/1600μg/mL). Behavioral assessments included thigmotaxis, vertical preference, and locomotor activity using automated tracking, with GABA/5-HT levels measured by ELISA. Compared with normal controls, caffeine-exposed zebrafish showed increased total movement time ( $P<0.05$ ), movement distance ( $P<0.05$ ), and large movement distance ( $P<0.05$ ), accompanied by reduced central zone time (-28%,  $P<0.05$ ) and distance (-31%,  $P<0.05$ ), with significantly decreased bottom-dwelling time ( $P<0.05$ ). YSNX administration (1600μg/mL and 550μg/mL) reversed these effects, significantly decreasing movement distance/time (vs caffeine group,  $P<0.05$ ) while increasing central zone exploration ( $P<0.05$ ). The high-dose group restored bottom-dwelling time to control levels ( $P<0.05$ ). Neurochemical analysis revealed caffeine-induced GABA reduction (vs normal group:  $P<0.05$ ) and 5-HT elevation ( $P<0.05$ ) in caffeine, YSNX-L, and YSNX-M groups. YSNX-H treatment increased GABA ( $P<0.05$  vs caffeine group) and decreased 5-HT ( $P<0.05$ ), while YSNX-M specifically reduced 5-HT ( $P<0.05$ ). These results demonstrate YSNX's ability to ameliorate anxiety-like behaviors in zebrafish, potentially through GABA/5-HT pathway modulation. This study provides experimental evidence supporting YSNX's therapeutic potential for anxiety disorders.

**Keywords:** Generalized anxiety; Yishen Ningxin formula; zebrafish;  $\gamma$ -aminobutyric acid; 5-hydroxytryptamine; behavioral Science

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## INTRODUCTION

Generalized anxiety disorder (GAD), a prevalent neuropsychiatric condition affecting 3-7% of global population, manifests through complex interactions between genetic predisposition, environmental stressors, and psychological vulnerability with frequent childhood onset (Creswell C *et al.*, 2021). The escalating prevalence (annual increase of 12-15% in industrialized nations) correlates with modern societal pressures, often co-occurring with insomnia (45-60% comorbidity) and depression (35% bidirectional linkage), creating self-perpetuating pathological cycles that severely impair cognitive function and life quality (Choi KW *et al.*, 2020). Current pharmacotherapy predominantly relies on benzodiazepines (e.g., alprazolam) and selective serotonin reuptake inhibitors (SSRIs) like sertraline. While effective short-term, these agents carry substantial limitations: benzodiazepines induce dependence within 4-6 weeks in 30-45% patients, and SSRIs exhibit delayed onset (2-4 weeks) with sexual dysfunction (40-60% incidence) (Suryawanshi O *et al.*, 2022). This therapeutic gap underscores the urgent need for non-addictive alternatives with faster action profiles (Wenhui L *et al.*, 2024). Emerging evidence implicates neurotransmitter dysregulation in GAD pathogenesis, particularly GABAergic

hypofunction ( $\leq 65\%$  normal levels in amygdala) and serotonergic imbalance (5-HT<sub>1A</sub> receptor downregulation  $\geq 28\%$ ) (Sharma P *et al.*, 2023, Borroto-Escuela DO *et al.*, 2021). Traditional Chinese Medicine (TCM) offers a well-tolerated alternative with multi-target mechanisms. The Yishen Ningxin Formula (YSNX), developed by Prof. Zhang Zhenxian (Shanghai University of TCM), demonstrates clinical efficacy in anxiety-related insomnia (72% response rate vs 58% for zolpidem in pilot trials), yet its GABA/5-HT modulation remains uncharacterized (Wenhui L *et al.*, 2024) Jianing S *et al.*, 2021). Zebrafish (*Danio rerio*) emerges as an optimal translational model for neurobehavioral studies, sharing 87% neurotransmitter pathway homology with humans (Kongli H *et al.*, 2023). Our study employs caffeine-induced anxiety models to: quantify YSNX's dose-responsive behavioral effects, elucidate GABA/5-HT pathway modulation, and establish preclinical evidence for TCM-based anxiety therapeutics.

## MATERIALS AND METHODS

### Materials

#### Animals

Wild-type AB zebrafish were used, and zebrafish in this experiment were provided and bred by Hangzhou Huite Biological Company. The breeding and reproduction were all completed on the platform. The experiment used larvae

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7 days after fertilization (7dpf). Screening and elimination criteria for zebrafish larvae: Strictly select the larvae 7 days after fertilization. The selection criteria are as follows: select the larvae with normal morphology, active motor behavior, and no malformations. Eliminate the individuals with developmental delay, malformations, abnormal motor behavior and obvious lesions. Breeding conditions: the temperature of the fish room is  $(28.5 \pm 1)^{\circ}$ , the light-dark condition is a 14h:10h cycle, and the pH of the fish water is 7.0-8.0. At this stage, zebrafish can obtain nutrients from their own yolk sac and do not need to be fed (Jianing S *et al.*, 2021).

### Drugs and reagents

Caffeine, Sigma, USA (Batch number: BCCC8969); MT, Solabio, Beijing (Batch number: 728Q024); GABA, 5-HT ELISA kit, Sideng Biotechnology, Shanghai (Batch number: m1064304V; m1065202V).

The Yishen Ningxin Formula (YSNX) contained six standardized components: *Astragalus membranaceus* (12g), *Ophiopogon japonicus* (12g), *Coptis chinensis* (5g), *Cinnamomum cassia* (5g), raw oyster shell (12g), and Pheretima (12g), prepared in accordance with patented protocols (Patent No: ZL2022114335310). Medicinal materials authenticated by Yueyang Integrated TCM-WM Hospital were subjected to optimized extraction: (1) Three-stage decoction with 5:1 water-herb ratio (30 min soaking, 1 hr initial boiling followed by two 1hr extractions), (2) Ethanol precipitation (99% anhydrous alcohol, 48 h at  $4^{\circ}\text{C}$ ), (3) Final concentration to 1g crude herb/mL through vacuum evaporation. Sterile filtration ( $0.22\mu\text{m}$ ) preceded aliquoting and storage at  $4^{\circ}\text{C}$  (Rui Z *et al.*, 2024).

### Main instruments

Behavioral analysis utilized the Noldus Danio Vision Tracking System (Netherlands). Biochemical assays employed: Beckman B37913 Centrifuge (USA); Thermo Fisher MK3117123001 Microplate Reader (USA); Likang CG-05 qPCR System (China); Sartorius 910324 Analytical Balance (Germany).

### Grouping

Wild-type AB zebrafish (7 days post-fertilization) were stratified into five cohorts ( $n=20/\text{group}$ ): Blank control (system water), Caffeine model ( $100\mu\text{mol/L}$ ), YSNX treatment: Low ( $200\mu\text{g/mL}$ ), Medium ( $550\mu\text{g/mL}$ ), High ( $1600\mu\text{g/mL}$ ).

### Modeling and intervention

#### Maximum tolerable concentration (MTC) of the drug

Serial dilutions of YSNX ( $50\text{--}1600\mu\text{g/mL}$ ) were evaluated in triplicate using 6-well plates ( $3\text{mL}/\text{well}$ , 24h exposure). Mortality thresholds established through iterative refinement (3 experimental cycles) confirmed  $1600\mu\text{g/mL}$  as MTC with  $<5\%$  acute toxicity (Rui Z *et al.*, 2024).

### Modeling and intervention methods

Five experimental cohorts were established: Blank control (untreated system water), Caffeine model ( $100\mu\text{mol/L}$ , 24h exposure), YSNX interventions: High ( $1600\mu\text{g/mL}$ ), Medium ( $550\mu\text{g/mL}$ ), Low ( $200\mu\text{g/mL}$ ) doses administered concurrently with caffeine challenge for 24h. Dose selection followed preliminary MTC determination ( $1600\mu\text{g/mL}$ ) using established fractionation protocols (Jing X *et al.*, 2020).

**Behavioral Assessments** Zebrafish movement patterns (7 dpf) were quantified using Etho Vision XT (Noldus) under infrared conditions. Continuous 1h recordings commenced 5h post-treatment, capturing total movement distance (cm) and duration (s) for 12 larvae per group (Rui Z *et al.*, 2024).

### Detection of thigmotaxis

Anxiety-like behavior was assessed in 24-well plates ( $15.88\text{ mm}$  inner diameter). Following 5min dark adaptation, zebrafish were subjected to 15min light-dark cycles (5min intervals). Automated tracking differentiated central (50% area) versus peripheral zones, with central occupancy (%) calculated as:  $(\text{Central duration} / \text{Total duration}) \times 100$ . See fig. 2 and fig. 3.

### New tank experiment

Vertical exploration was monitored in rectangular tanks ( $40 \times 18 \times 20.5\text{cm}$ ) filled to 15cm depth. After 5-10min acclimation, 60min recordings quantified bottom-dwelling time (%) and vertical transitions using Viewpoint tracking (Lucon-Xiccato T *et al.* 2023).

### Detection of gaba, 5-HT content

Post-treatment zebrafish ( $n=60/\text{group}$ ) were homogenized in ice-cold PBS ( $1:9\text{w/v}$ ) and centrifuged ( $10,000 \times g$ , 15min,  $4^{\circ}\text{C}$ ). Supernatant GABA/5-HT levels were determined via ELISA (Human ELISA Kit #AB1234), following manufacturer protocols with modifications:

1. Standards/samples ( $25\mu\text{L}$ ) loaded in triplicate.
2. BCA working solution incubation ( $200\mu\text{L}/\text{well}$ ,  $37^{\circ}\text{C}$ , 30min).
3. Absorbance read at  $562\text{nm}$  (Thermo Fisher MK3117123001).
4. Concentrations extrapolated from standard curves ( $R^2 > 0.99$ ) (Rui Z *et al.*, 2024).

### Ethical Approval

All experimental animal protocols in this study were reviewed and approved by Shanghai University of Traditional Chinese Medicine Affiliated Yueyang Hospital of Integrated Traditional Chinese and Western Medicine Animal Ethics Committee, (Approval Letter No. YYLAC-2023-2N, dated 25/07/2024), and all experimental animal protocols were carried out by following the guidelines of (Ethical review guidelines for laboratory animal welfare in China).

## STATISTICAL ANALYSIS

Data normality was verified using Shapiro-Wilk tests ( $\alpha=0.05$ ) with homogeneity of variance confirmed by Levene's test. One-way ANOVA with Tukey post-hoc analysis compared group differences (Graph Pad Prism 8.0). Results expressed as mean  $\pm$  SD. Significance thresholds:  $P < 0.05$  vs blank control,  $P < 0.01$  vs caffeine model.

## RESULTS

### Maximum tolerable concentration of the drug

According to the initially set 6 groups of concentrations: 50ug/ml, 250ug/ml, 500ug/ml, 800ug/ml, 1150ug/ml, 1600ug/ml, no zebrafish died in each group, so the medication concentration was increased to 1200ug/ml, 1600ug/ml, 2000ug/ml, 2400ug/ml, 2800ug/ml, 3200ug/ml, respectively. When the medication concentration was 1600ug/ml, all 10 zebrafish survived. When the medication concentration was 2000ug/ml, one zebrafish was in poor health and finally died. When the medication concentration was 2400ug/ml, 2 zebrafish were in poor health and finally died. Therefore, the final MTC of Yishen Ningxin Formula was determined to be 1600ug/ml. In the following Yishen Ningxin Formula medication experiment, this maximum tolerable concentration was set as the high concentration of Yishen Ningxin Formula, the maximum tolerable concentration was set as the medium concentration of Yishen Ningxin Formula (550ug/ml) and 1/9 of the maximum tolerable concentration was set as the low concentration of Yishen Ningxin Formula (200ug/ml) See table 1.

### Effect on autonomous activity

Compared with the blank group, the movement time and distance of zebrafish larvae in the caffeine group significantly increased, and the large movement distance increased ( $P < 0.05$ ). Compared with the blank group, the movement time and distance of zebrafish larvae in the Yishen Ningxin Formula high-dose group decreased ( $P < 0.05$ ). Compared with the caffeine group, the movement distance and time of the Yishen Ningxin Formula high, medium, and low dose groups decreased ( $P < 0.05$ ). Compared with the blank group, the large movement distance of the Yishen Ningxin Formula medium and high dose groups decreased ( $P < 0.05$ ). See fig. 1.

### Effect on thigmotaxis

Compared with the blank group, the percentage of time spent in the central area and the percentage of distance traveled in the central area in the caffeine group were significantly reduced ( $P < 0.05$ ). When compared with the blank group, there was no significant difference in the percentage of time spent in the central area and the percentage of distance traveled in the central area in the

high-dose Yishen Ningxin Formula group ( $P > 0.05$ ). Compared with the caffeine group, there were significant differences in the percentage of time spent in the central area and the percentage of distance traveled in the central area in the medium- and high-dose Yishen Ningxin Formula groups ( $P < 0.05$ ). When compared with the caffeine group, there was no significant difference in the percentage of time spent in the central area and the percentage of distance traveled in the central area in the low-dose Yishen Ningxin Formula group ( $P > 0.05$ ) See table 2 and table 3.

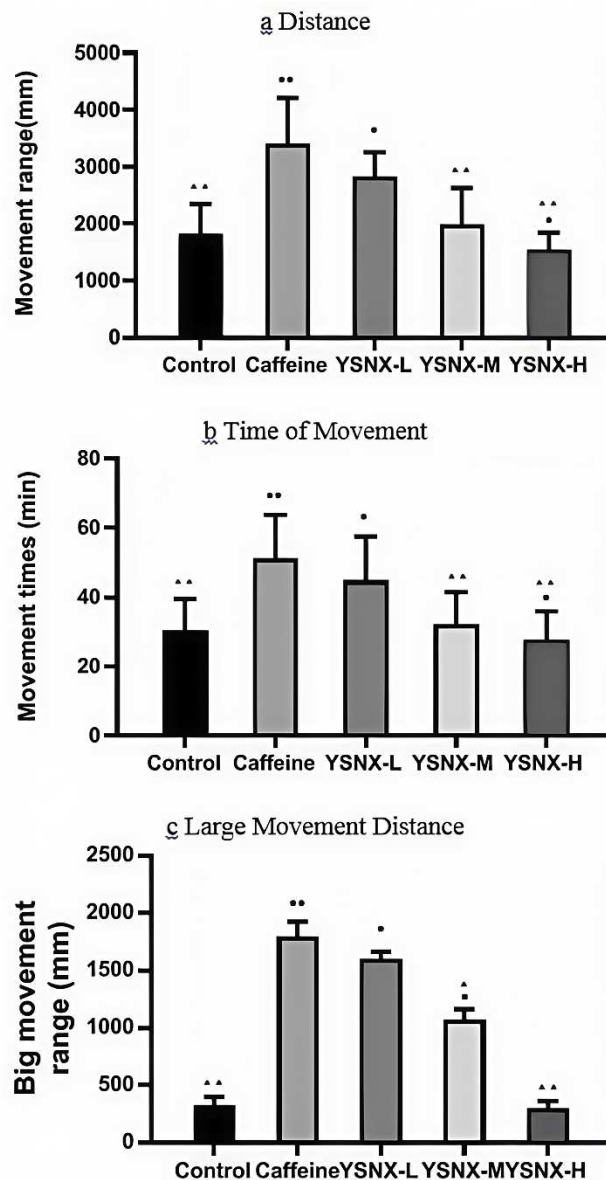


Fig. 1: Comparison of Zebrafish Behavior in Each Group

### New tank experiment

The zebrafish new tank experiment larvae movement trajectory map is shown in fig.4. With the addition of caffeine, the zebrafish stay time in the bottom area is prolonged. Compared with the blank group, the bottom

stay time of the zebrafish in the caffeine group decreased ( $P<0.05$ ); Compared with the caffeine group, the bottom stay time of the blank group and the Yishen Ningxin Formula medium and high dose groups decreased ( $P<0.05$ ).

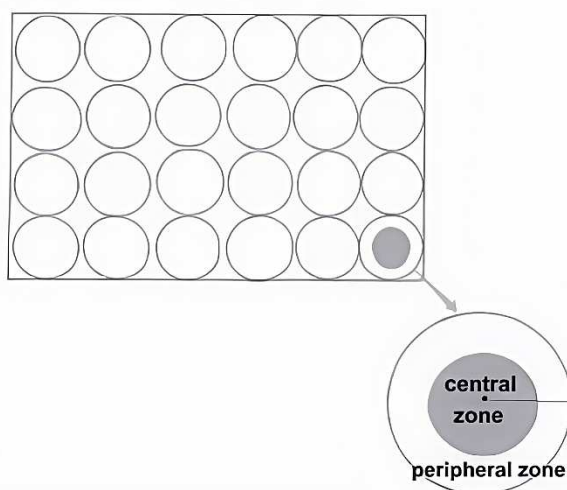


Fig. 2: Observation of Zebrafish Thigmotaxis

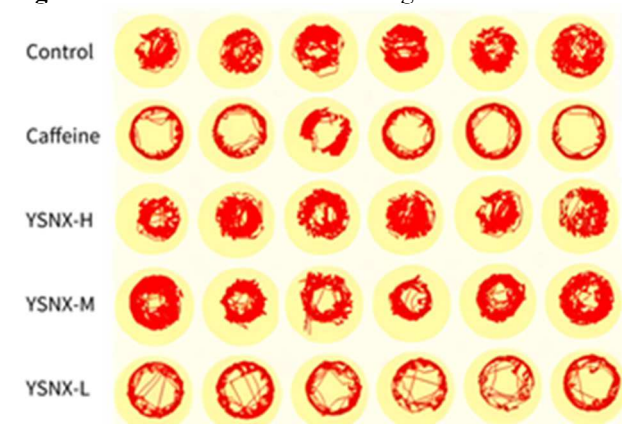


Fig. 3: Zebrafish Thigmotaxis Behavior Trajectory Map (The red line represents the movement trajectory of zebrafish larvae within 1 hour)

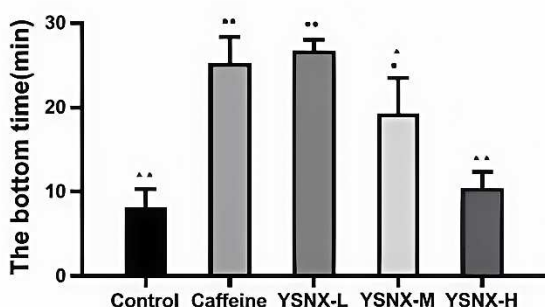


Fig. 4: Bottom Stay Time in the New Tank Experiment

#### Effect on gaba, 5-HT content

Compared with the normal group, the GABA content of the caffeine group and the Yishen Ningxin Formula low and

medium dose groups decreased, and the 5-HT content significantly increased ( $P<0.05$ ). Compared with the caffeine group, the GABA content of the Yishen Ningxin Formula high-dose group significantly increased, and the 5-HT content significantly decreased ( $P<0.05$ ); Compared with the caffeine group, the 5-HT content of the Yishen Ningxin Formula medium-dose group decreased, ( $P<0.05$ ) See table 4 and table 5.

## DISCUSSION

Generalized anxiety (Fagan HA *et al.*, 2024) (generalized anxiety disorder, GAD), also known as generalized anxiety disorder, belongs to the category of neurosis. GAD is characterized by frequent, persistent tension and worry, accompanied by significant insomnia, lack of concentration, irritability, muscle tension, etc. According to its symptom manifestation, it can be attributed to the categories of “depression” and “organ irritability” in traditional Chinese medicine, and the onset of the disease is related to liver qi stagnation, spleen loss of movement, and heart spirit malnutrition (Jiepeng O *et al.*, 2024). The research group understands depression from the perspectives of “qi” and “spirit”, and believes that the onset of GAD belongs to the disorder of qi movement, and the clinical treatment of insomnia focuses on regulating qi and blood and balancing yin and yang. Yishen Ningxin Formula is Professor Zhang Zhenxian’s experience in treating insomnia and anxiety. The core drug Astragalus is sweet and warm to benefit qi, nourish the spirit, and nourish the five internal organs, as the monarch. Ophiopogon “replenishes the heart qi’s labor injury... nourishes the essence and strengthens the yin”, as the minister. It is supplemented by earth dragon to calm the shock, relieve annoyance, and calm the mind, and also activate blood and remove stasis; oyster calms the mind and nourishes the yin and hides the yang; coptis removes annoyance, can treat insufficient yin blood leading to heart fire deficiency and hyperactivity; cinnamon helps yang to warm the water organs and stimulate the growth of qi and blood. The previous research of the research group (Rui Z *et al.*, 2023) Yishen Ningxin Formula has the effect of improving the sleep condition of insomnia patients and accompanying negative emotions such as anxiety and depression, and also has a good circadian rhythm regulation effect on hormone levels such as melatonin. Zebrafish has a monoamine nervous system, which has many similarities with the nervous system of vertebrates. The neurotransmitter systems such as 5-hydroxytryptamine and norepinephrine in its body are highly similar to mammals, and it has no brain dopamine neurons like mammals (Zahrai A *et al.*, 2020). Zebrafish, like humans, use cortisol as a stress response hormone, while rodents use corticosterone as the main stress response hormone (Faught E *et al.*, 2022). In view of these physiological characteristics of zebrafish, it has become a model organism with more research value than rodents.

**Table 1:** Drug concentration table

Drug	drug concentration μg/ml	Total zebrafish	Number of zebrafish deaths	Zebrafish survival rate (%)
Yishen Ningxin Formula	1200	20	0	100
	1400	20	0	100
	1600	20	0	100
	2000	20	1	95
	2400	20	5	75
	2800	20	20	0
	3200	20	20	0

**Table 2:** Percentage of Time in the Central Area ( $\bar{x} \pm s$ )

Group (n=12)	Percentage (%)
Control	38%±6%▲
Caffeine Group	11%±3%*
YSNX-L	8%±1%*
YSNX-M	25%±2%*▲
YSNX-H	39%±3%▲

**Table 3:** Percentage of Distance in the Central Area ( $\bar{x} \pm s$ )

Group (n=12)	Percentage (%)
Control	63%±4%▲
Caffeine Group	23%±4%*
YSNX-L	25%±5%*
YSNX-M	30%±2%*▲
YSNX-H	46%±5%▲

**Table 4:** GABA Secretion Level ( $\bar{x} \pm s$ )

Group (n=12)	GABA Secretion Level (pg/mg)
Control	32.5±2.2▲
Caffeine Group	16.8±0.9*
YSNX-L	11.2±1.8*
YSNX-M	17.0±2.6*
YSNX-H	24.5±0.5▲

**Table 5:** 5-HT Secretion Level ( $\bar{x} \pm s$ )

Group (n=12)	5-HT Secretion Level (pg/mg)
Control	27.3±4.3▲
Caffeine Group	36.7±2.2*
YSNX-L	39.1±3.0*
YSNX-M	27.6±2.4*▲
YSNX-H	28.9±1.6▲

Caffeine is one of the commonly used drugs to create animal anxiety models (Neri D *et al.*, 2019). Related research (Bartoszek A *et al.*, 2024) found that after caffeine treatment, zebrafish larvae showed an increase in the duration of movement and a large movement coverage distance and a decrease in the duration and coverage distance of the resting state, similar to the clinical characteristics of anxiety.

The behavioral research results of this experiment are similar to the above research results. At a caffeine concentration of 100μmol/L, the zebrafish anxiety model was successfully replicated. Yishen Ningxin Formula can improve the abnormal activity state of zebrafish and improve anxiety-like behavior changes after caffeine modeling by reducing the movement distance and movement time of zebrafish anxiety model, and its related effects become more obvious with the increase of concentration. Thigmotaxis can reflect the anxiety behavior of zebrafish. The visual response test of light-dark alternation can cause hyperactivity of zebrafish larvae. During the light-dark transition, the thigmotaxis behavior of zebrafish larvae increases, reflecting that this external environmental stimulus increases the anxiety level of zebrafish larvae (Ziwen C *et al.*, 2021). At the same time, caffeine can induce anxiety-like responses in zebrafish, and it is related to dose and collective environment (Li Y, *et al.*, 2024). The results of this study show that under the stimulation of light and dark, the percentage of time and distance in the central area of the caffeine group decreased, suggesting that the thigmotaxis behavior of zebrafish increased, which is consistent with the characteristics of zebrafish anxiety behavior. The thigmotaxis behavior of zebrafish in the Yishen Ningxin Formula high-dose group decreased compared with the caffeine group, suggesting that Yishen Ningxin Formula can reduce the anxiety level of zebrafish larvae during caffeine modeling under 15min light-dark alternation stimulation. In the new tank diving experiment, zebrafish usually stay at the bottom for a long time after entering the new tank, and the time spent at the top is reduced, indicating a high degree of anxiety (De Abreu MS *et al.*, 2021). Using this anxiety model, the anti-anxiety effect of drugs can be evaluated. The results of this experiment using the new tank diving experiment show that under the action of 100μmol/L caffeine, the anti-anxiety effect is obvious, the caffeine group larvae stay at the bottom of the tank for a long time, and the bottom stay time of the zebrafish in the Yishen Ningxin Formula medium and high dose groups compared with the caffeine group Reduced, suggesting that Yishen Ningxin Formula can alleviate the anxiety of zebrafish larvae during caffeine modeling.



In caffeine modeling, we noticed the changes in GABA and 5-HT neurotransmitter content. Previous studies (Jones BE, 2020, ZZ *et al.*, 2021) have found that caffeine can inhibit GABA activity and down-regulate GABA receptors. In sleep deprivation animal experiments (DF Y *et al.*, 2023, Suganya K *et al.*, 2020), there are reports of increased or decreased 5-HT, both of which can affect anxiety. However, after short-term application of caffeine modeling, the levels of 5-HT and its metabolite 5-HIAA in zebrafish increased (Gutierrez HC *et al.*, 2020) Kazanskaya RB *et al.*, 2025). The results of this study are consistent with other drug screening mechanisms. The results show that after 100µmol/L caffeine modeling, the GABA content in zebrafish larvae decreases and the 5-HT content increases, which has a calming and hypnotic effect on insomnia zebrafish and reduces anxiety. In summary, caffeine modeling can cause an increase in zebrafish movement behavior and changes in anxiety behavior. The results of this study suggest that the behavioral changes caused by caffeine are closely related to abnormal GABA and 5-HT content. Yishen Ningxin Formula can significantly improve the behavioral characteristics of zebrafish after caffeine modeling and improve anxiety-like behavior changes, and can make the levels of GABA and 5-HT related disorders in zebrafish larvae tend to be normal, reflecting the multi-target effect of traditional Chinese medicine treatment, which can provide experimental evidence for clinical treatment of insomnia drugs. It lays the foundation for further in-depth research and exploration of the anti-anxiety and anti-depression mechanism of Yishen Ningxin Formula.

## CONCLUSION

In this experiment, by dividing wild-type AB zebrafish at 7 days post-fertilization into different groups, establishing an anxiety model, and conducting various behavioral tests and detecting the contents of neurotransmitters, it was found that Yishen Ningxin Formula can improve the anxiety behaviors of zebrafish larvae. Its mechanism may be related to the regulation of the disorders of gamma-aminobutyric acid (GABA) and 5-hydroxytryptamine (5-HT). This provides ideas for further research on the treatment of anxiety-related diseases with traditional Chinese medicine.

## Conflict of interests

All authors disclose no relevant conflict of interests.

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