

An *In Vivo* Assessment: Cardiotoxicity Induced by Three Kinds of Addictive Drugs (Methamphetamine, Ketamine, and Methadone) in Zebrafish Embryos

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Abstract

This study aimed to observe cardiotoxicity induced by three kinds of addictive drugs (i.e., methamphetamine (METH), ketamine (KT), and methadone) in zebrafish embryos. Zebrafish embryos were exposed to various doses of METH, KT, and methadone solutions. The qualitative and quantitative cardiotoxicities were recorded at 24 h and 48 h posttreatment using specific endpoints, i.e., heart rate, heart malformation, pericardial edema, and circulation abnormalities. Results showed that METH at 500 mg/L dose induced a heart rate decline and pericardial edema at 24 h posttreatment, whereas a 1,000 mg/L dose induced significant cardiac malfunctions (i.e., heart rate, pericardial edema, and circulation decrease/absence) at 12 h and 24 h posttreatment compared with the control group. The heart rate of zebrafish embryos treated with KT at 500 mg/L dose decreased compared with the control groups at 24 h posttreatment, whereas a minimum dose of 1,000 mg/L induced significant cardiac malfunctions (i.e., heart rate, pericardial edema, and circulation decrease/absence) at 12 h and 24 h posttreatment. All of the zebrafish embryos treated with methadone at a minimum dose of 500 mg/L died, whereas methadone at 200 mg/L and 300 mg/L dose induced significant cardiac malfunctions (i.e., heart rate, pericardial edema, and circulation decrease/absence) at 12 h and 24 h posttreatment. The results confirm the potential cardiotoxicity of the three kinds of addictive drugs (i.e., METH, KT, and methadone), particularly of methadone. Therefore, more attention must be focused on the risk of clinical application of methadone.

Keywords: Addictive drugs; Methamphetamine; Ketamine; Methadone; Zebrafish embryos; Cardiotoxicity

Introduction

Methamphetamine (METH) is a highly addictive stimulant drug that activates certain systems in the brain. METH also causes its toxic effects on the central nervous system to be more pronounced, resulting in serious worldwide public health problems. METH abusers often show extreme paranoia, depression, and anxiety, and 36% of METH addicts suffer from psychiatric disorders [1]. Ketamine (KT) is a noncompetitive N-Methyl-D-aspartate (NMDA) receptor antagonist introduced as an anesthetic agent and is widely used as a veterinary anesthetic. However, KT has been used as a club drug since the 1970s. The inappropriate use of KT, which may cause potent pharmacological effects and serious health problems, has been extensively reported in recent years [2]. Furthermore, NMDA receptor antagonists can cause severe behavioral disorders and deficiencies [3]. Methadone is a long-acting opiate agonist and has been used as the main agent for the detoxification of heroin-dependent patients. Many studies have confirmed the efficiency of methadone maintenance therapy (MMT) in improving the rehabilitation of intravenous opiate-addicted patients as it decreases illicit drug consumption and criminal behavior. However, MMT is associated with several problems, which includes limited patient and community acceptance. The treatment seems to be independent of the methadone dose, which does not decrease with the length of improvement [4]. In addition, high doses of methadone can lead to various side effects.

Zebrafish (*Danio rerio*) is a small freshwater teleost species, reaching only 3 cm in length. During the embryonic and larval stages, zebrafish is only 1 mm to 4 mm long. Zebrafish larva can live for seven days in a single well of a standard 96-well or 384-well plate, surviving on nutrients stored in the yolk sac [5]. The zebrafish larva develops rapidly, and its organogenesis is complete within 48 h post-fertilization

(hpf). The heart of the zebrafish begins beating after 26 hpf and begins looping by 48 hpf. The heart is wrapped by a pericardial sac in the thoracic cavity under the atrium and the pectoral bone [6]. The relevance and predictability of drug response between zebrafish and humans have been investigated, and previous studies have reported that zebrafish shows high percentage predictability (78% and 70%) of drug response [7,8]. In addition, genes identified in the original zebrafish organogenesis screens have been consistently validated in humans or mice [5]. The genes possess orthologs for >70% of human genes and >86% of 1,318 human drug subjects. From simple investigations, heart rate responses have been established to have an excellent correlation with known adult human repolarization cardiac toxicity, and clinically relevant drug to drug interactions have been recapitulated. Thus, the zebrafish has become an important and well-known model in biology, particularly in fields such as genetics, pharmacology, environmental science, preclinical medicine, DNA damage repair, and drug discovery [9,10], where it is used to understand the effects of certain toxins and drugs on cardiovascular regulation.

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Organ-specific toxicity remains to be the most common reason for the failure of drug therapy, prevention of the clinical use of drugs, and drug withdrawal from the market [11]. Therefore, a rapid and precise estimation of such toxicity risk is needed for drug use. As research on the toxicity of the drugs has mostly concentrated on mice and cells, we chose the zebrafish embryos as models in this study to further evaluate the toxicity of the three addictive drugs (METH, KT, and methadone). This study is the first to report on the toxicity of the three addictive drugs [12].

Materials and Methods

Animals

Adult wild-type zebrafish (*D. rerio*, AB strain) were obtained from the Key Laboratory of Zebrafish Modeling and Drug Screening for Human Diseases of Guangdong Higher Education Institutes, Southern Medical University (Guangzhou, China). The zebrafish were maintained under a 14:10 h cycle (lights on at 8 am, lights off at 6 pm) in an automatic circulating tank system and fed live brine shrimps once a day. The water temperature was maintained at approximately $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$, and pH was maintained at 7.0 ± 0.5 . The day before spawning, two pairs of adult zebrafish were placed in a breeding tank equipped with a spawning tray. After spawning, embryos were maintained at 28°C in fish water (0.2% instant ocean sea salt in deionized water, pH 7.2) [13]. Fertilized embryos staged at 48 hpf were selected and washed for later use. All experimental protocols and animal handling procedures were performed in accordance with the National Institutes of Health (Bethesda, MD, USA) Guide for the Care and Use of Laboratory Animals and were approved by the Experimental Animal Ethics Committee of Southern Medical University [14].

Reagents

Methamphetamine hydrochloride (No. 1212-9802) was obtained from the National Narcotics Laboratory, China. Ketamine hydrochloride (50 mg/mL) was purchased from Jiangsu Hengrui Medicine Co., Ltd., China. Methadone (raw material powder) was provided by Tianjin Central Pharmaceutical Co. Ltd. (No. 020111). Methamphetamine hydrochloride, ketamine hydrochloride, and methadone were dissolved in fish embryo medium to their final concentrations before use.

Treatment of zebrafish embryos with methamphetamine, ketamine, and methadone

The 48 hpf manually fertilized embryos were used for the treatment of different concentrations of METH, KT, and methadone. From the preliminary experimental data (data not shown), embryos were exposed to METH solutions of 100, 200, 300, 500, and 1,000 mg/L, KT solutions of 250, 500, 1,000, and 2,000 mg/L, and methadone solutions of 100, 200, 300, 500, and 1,000 mg/L continuously for 12 h and 24 h. In each treatment, 30 embryos were distributed into six-well plates (Nest Biotech, China) with 3 mL each of the three drugs. Control groups were treated with an equivalent amount of embryo water. During the experimental duration, the embryos were examined daily and dead embryos were removed to prevent contamination among the surviving embryos [15].

Scoring heart rate

Embryos in six-well plates were subjected to visual observation and image acquisition using a stereomicroscope (Olympus, Tokyo, Japan) connected to a digital camera (Canon, Tokyo, Japan) [16]. The heart

rate of each embryo was measured by visually counting the ventricular beats per minute on the live video using a timer (stop watch). This process was replicated three times within the same stage of zebrafish embryos [15].

Cardiovascular toxicity assessment

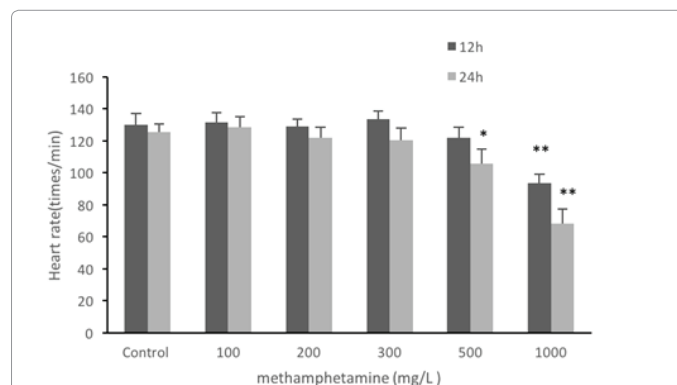
Different dosages of the three addictive drugs were used independently to assess the cardiovascular toxicity of zebrafish embryos. After the treatment of zebrafish embryos from 12 hpf to 24 hpf, 10 zebrafish from each group were selected randomly for visual observation and image acquisition of specific phenotypic endpoints under the dissecting stereomicroscope. The occurrence of pericardial edema, abnormal circulation (decrease and absence), hemorrhage, and thrombosis were evaluated by double-blind observation [17].

Results

Methamphetamine induces changes in heart rate and cardiotoxicity in zebrafish embryos

As illustrated in Figure 1, the heart rate of zebrafish embryos treated with various doses of METH changed at 12 h and 24 h posttreatment. Doses of up to 500 mg/L did not have any significant effect at 12 h posttreatment. However, a longer time can reduce the heart rate noticeably compared with the control at 24 h posttreatment ($p < 0.05$). By contrast, doses of up to 1,000 mg/L significantly reduced the heart rate at 12 h and 24 h posttreatment ($p < 0.01$).

Cardiovascular toxicity-associated morphological abnormalities were observed and shown in Figure 2. In the control groups (Figure 2D), no morphological changes were observed in the heart and pericardium of zebrafish embryo. Embryos treated with METH at 500 mg/L exhibited pericardial edema, hemorrhage, and morphological abnormalities at 24 h posttreatment (Figure 2A1). Zebrafish embryos in groups treated with 1,000 mg/L of METH exhibited severe manifestations of cardiac and pericardial abnormalities, such as heart malformations and cardiac looping defects at 12 h posttreatment (Figure 2A2). In particular, at 24 h posttreatment, the pericardium of zebrafish embryos was enlarged and filled with turbid fluid, which was three times larger than that of the control group (Figure 2A3).



*significance was set at $p < 0.05$; **significance was set at $p < 0.01$.

Figure 1: Effect of methamphetamine (METH) on the heart rate of zebrafish embryos. Embryos were exposed to various doses (100 mg/L to 1,000 mg/L) of METH. Each exposure group consisted of 10 embryos, and the experiment was repeated at least three times. The heart rate at 12 h and 24 h post-exposure was measured in beats per minute. The data from all of the embryos in a group were averaged and shown as the mean $\pm$ SD. One-way ANOVA was used to compare the heart rates between control and treated groups.

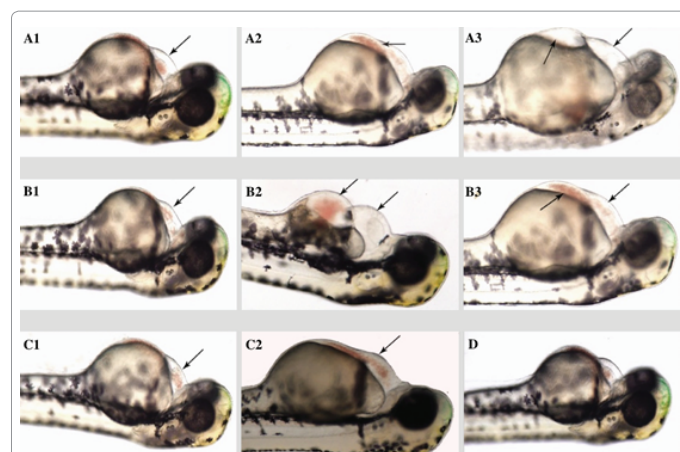


Figure 2: Representative images of zebrafish in the embryonic cardiac visual analysis: A) Treatment of METH (A1: 500 mg/ml 24 h posttreatment; A2: 1,000 mg/ml 12 h posttreatment; A3: 1,000 mg/ml 24 h posttreatment). B) Treatment of KT (B1: 500 mg/ml 24 h posttreatment; B2: 2,000 mg/ml 12 h posttreatment; B3: 2,000 mg/ml 24 h posttreatment). C) Treatment of methadone (C1: 100 mg/L 24 h posttreatment; C2: 300 mg/L 24 h posttreatment). D) Control (24 h posttreatment).

Ketamine induces changes in heart rate and cardiotoxicity in zebrafish embryos

As illustrated in Figure 3, the heart rate of embryos treated with various doses of KT was altered at 12 h and 24 h posttreatment. Doses of up to 500 mg/L caused no significant difference at 12 h posttreatment. By contrast, the heart rate was significantly reduced compared with the control group at 24 h posttreatment ($p < 0.01$). Doses of up to 1,000 mg/L and above significantly reduced the heart rate at 12 h and 24 h posttreatment.

At 2,000 mg/L, the embryos treated with KT showed severe pericardial edema, weak pigmentation, hemorrhage, and morphological abnormalities at 12 h (Figure 2B2) and 24 h (Figure 2B3) posttreatment. In addition, a part of the embryos exhibited specific deformities and circulation decrease and absence at 24 h posttreatment with 500 mg/L (Figure 2B1).

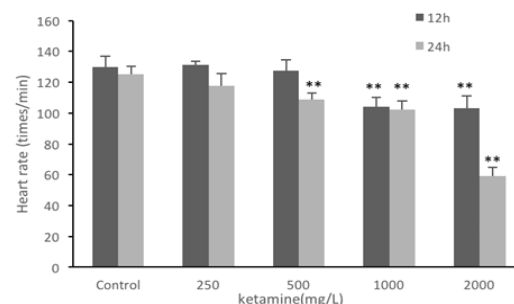
Methadone induces changes in the heart rate and cardiotoxicity in zebrafish embryos

As illustrated in Figure 4, doses of up to 200 mg/L and above significantly decreased the heart rate of the embryos compared with the control group, which exhibited an acute and strong cardiotoxicity. All zebrafish embryos at 500 and 1,000 mg/L died at 12 h post-methadone treatment.

With the increase in the dose of methadone treatment, increasingly severe manifestations of cardiac and pericardial abnormalities were observed in the embryos. In addition, almost all of the zebrafish showed circulation abnormalities. Bi-pericardial edema was also observed in the embryos at 24 h posttreatment with 100 mg/L (Figure 2C1). In groups treated with 300 mg/L of methadone, the zebrafish pericardium was enlarged, filled with turbid fluid, and had strong pigmentation compared with the control groups (Figure 2C2).

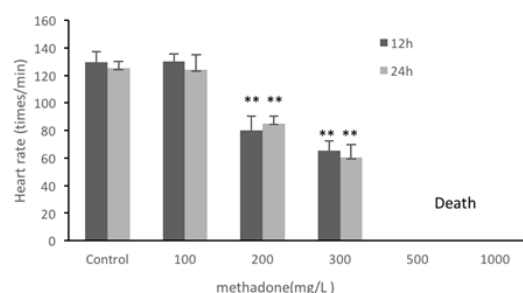
Discussion

Drug addiction is a chronic and relapsing medical condition that presents a high burden to the individual and society. Addiction produces extensive medical and psychosocial consequences. METH



*significance was set at $p < 0.05$; **significance was set at $p < 0.01$.

Figure 3: Effect of ketamine (KT) on the heart rate of zebrafish embryos. Embryos were exposed to various doses (250 mg/L to 2,000 mg/L) of KT. Each exposure group consisted of 10 embryos, and the experiment was repeated at least three times. The heart rate at 12 h and 24 h post-exposure was measured in beats per minute. The data from all of the embryos in a group were averaged and shown as the mean $\pm$ SD. One-way ANOVA was used to compare the heart rates between control and treated groups.



*significance was set at $p < 0.05$; **significance was set at $p < 0.01$.

Figure 4: Effect of methadone on the heart rate of zebrafish embryos. Embryos were exposed to various doses (100 mg/L to 1,000 mg/L) of methadone. Each exposure group consisted of 10 embryos, and the experiment was repeated at least three times. The heart rate at 12 h and 24 h post-exposure was measured in beats per minute. The data from all of the embryos in a group were averaged and shown as the mean $\pm$ SD. One-way ANOVA was used to compare the heart rates between control and treated groups.

and KT are two highly addictive drugs that are severely abused. By contrast, methadone is often used as a substitution therapy for drug addiction, which causes a strong addiction to the said drug.

Previous research on the toxicity of the drugs have mostly been concentrated on mice or cells that have a high throughput testing of cytotoxicity and exhibit high similarity with human diseases. However, mice and cells remain to have disadvantages, such as being expensive and time-consuming. By contrast, zebrafish have many advantages compared with the traditional experimental animals and cell lines. The most important advantage of using zebrafish is that it can develop normally without any functional blood circulation for up to a week wherein major developmental processes are completed [18]. Afterward, the researcher can continue to conduct the experiments, which could not be accomplished with animals that require cardiac function for survival and development. Thus, in this study, we chose the zebrafish embryos as the model to evaluate drug-induced cardiotoxicity further.

The toxic effects of these drugs on zebrafish embryonic development were analyzed. Accordingly, the therapeutic and toxic responses for both cardiac and non-cardiac drugs can be assessed by simply

immersing the embryos in the water with the test drug [19,20] and the zebrafish cardiotoxicity analysis is reliable and valid. Cardiac functions such as heart rate, contractility, rhythmicity and gross morphology can be evaluated using live and whole mount staining because zebrafish are transparent [21]. Moreover, the zebrafish larva was shown to be a good model for the investigations on drug toxicity, the annotation of small molecule function, and the empirical pathway discovery in genetic disease. Several groups have explored its utility for the prediction of cardiotoxicity. Using simple heart rate responses, researchers were able to establish an excellent correlation with the known human adult repolarization cardiac toxicity and to recapitulate clinically relevant drug-to-drug interactions, which have the potential to strengthen the human cardiovascular system. As demonstrated by our results, at 12 h and 24 h of treatment, METH at 1,000 mg/L could significantly decrease the heart rate of the embryo and induce cardiac and pericardial malformation. The treatment with KT decreased the heart rate in zebrafish, followed by visible observations of the morphology of the heart, embryonic toxicity, and decreased blood accumulation in the heart area. In addition, the treatment with methadone at doses of 500 mg/L and 1,000 mg/L left all zebrafish embryos dead. Therefore, methadone is confirmed to cause the most serious cardiotoxicity to zebrafish embryos among the three drugs. We suggest that doctors and patients should be more cautious with MMT. Methadone-treated patients are also advised to have a periodic inspection of the cardiovascular system [12]. Furthermore, future experiments are required to explore the exact mechanism of how these addictive drugs regulate the heart rate in zebrafish (Figures 5-7).

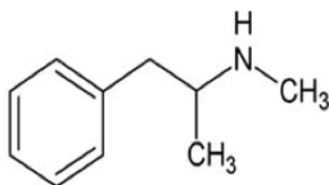


Figure 5: The structure of methamphetamine.

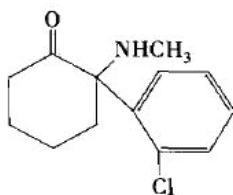


Figure 6: The structure of ketamine.

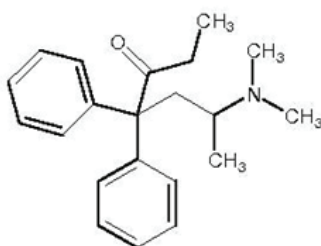


Figure 7: The structure of methadone.

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