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Comparative neurotoxicity study of mercury-based inorganic compounds including Ayurvedic medicines Rasasindura and Kajjali in zebrafish model

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ABSTRACT

Zebrafish behavioral model is a powerful tool for neuroscience research. Behavioral changes in the zebrafish are studied by administering drugs. With the aid of automated and open-source MATLAB program, high-accuracy tracking of zebrafish can be achieved, and the important behavioral parameters can be calculated. Although mercury is accepted as a potent neurotoxin, used as a key element for preparing certain Ayurvedic medicines. In this work, mercury-based inorganic compounds, including $HgCl_2$, HgS, and Ayurvedic medicines (Rasasindura and Kajjali) were administrated in zebrafish, and the effects on various behavioral parameters and cortisol levels were studied. A significant change in the basic locomotor parameters of fish was observed including speed (43% reduction), meander (150% increment), and a number of freeze points (125% increment), during 5-day treatment of $HgCl_2$ along with a 3-fold increase in cortisol level against the control groups. Abnormal behavior was also recorded in color preference test, and novel tank diving behavior of $HgCl_2$-treated groups, which can be attributed to the neurotoxicity induced by the $HgCl_2$ administration. Contrary to this, the Rasasindura-treated group showed a significant increase in speed by 33%, decrease in meander by 20%, decrease in freeze points by 30%, and insignificant alteration in cortisol levels, which can be related to the rejuvenating nature of the Ayurvedic medicine Rasasindura. Additionally, Kajjali treated group did not show any substantial changes in zebrafish cortisol level and behavioral parameters except one in the diving test that indicates lowering stress. Similarly, HgS group showed normal behaviors except two irregular motor behaviors identical with the $HgCl_2$ group. From these results, it can be concluded that the mercury-based Ayurvedic Rasasindura and Kajjali did not show any adverse effect or toxicity on zebrafish behavior model.

1. Introduction

Zebrafish (*Danio rerio*) model is becoming a very popular tool for drug screening, behavioral research, toxicity study, and translational neuroscience (Kalueff et al., 2013; Penberthy et al., 2002). Zebrafish, being a lower vertebrate, have almost 70% homologous human diseases gene (Santoriello and Zon, 2012) has many advantages as model organism including low cost, easy to maintain, plenty of offspring within a shorter time, transparent embryo, and complex brain function (Gerlai, 2012). As neuroanatomy of zebrafish is parallel with that of the humans (Kalueff et al., 2013; Panula et al., 2010), it is used to easily model complex brain disorders. The robust behavior of zebrafish is controlled by the nervous system, so any neurological changes are reflected in the behavior of zebrafish. Therefore, behavioral assessment of zebrafish provides numerous opportunity to study the various neurotoxic exposure. Although all behavioral parameter changes are not neurological, sometimes musculoskeletal issues can affect the behavior (Tierney, 2011). Anxiety and stress driven behavior in fish can be evoked by novelty (Cachat et al., 2010; Egan et al., 2009). Alteration of locomotor parameter including average speed, meander, freezing duration, and behavioral phenotype can be induced by anxiolytic or anxiogenic substances. For the behavioral studies, automated video tracking method enables for high throughput screening in the zebrafish model.

Hg and its compounds cause toxic effects on organs like kidney, lungs, skin, and eye, nervous and immune systems (Rice et al., 2014). One of the worst examples of Hg-poisoning was in Japan in the middle of the twentieth century, which caused the death of thousands of victims due to the consumption of Hg-contaminated seafood (Takeuchi et al., 1962). The disease is known as Minamata disease (Harada, 1995), a neurological syndrome. The recent cases of Hg-toxicity in Iran (Hg-contaminated wheat) and USA (Thimerosal used for vaccine preservative) (Gochfeld, 2003) create great concern about the uses of mercury.

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However, various forms of inorganic mercury compounds (mainly mercury sulfide, HgS) have been used in Indian traditional medicine (Ayurveda), and Chinese traditional medicine since time immemorial (Williamson, 2004). In recent times, many Hg-based medicines including Rasasindura, Kajjali, and Makardhwaj are commonly used in Ayurveda to treat various types of chronic diseases such as syphilis, pleural effusion, high fever, and nervous disorders (Kamath et al., 2012; Singh et al., 2009). The efficacy of these Hg-based Ayurvedic medicines can be attributed to their immunomodulatory action (Sinyorita et al., 2011). These drugs are believed to be antioxidant and used as the rejuvenating agent (Gholap et al., 2015; Singh et al., 2009). Although the Ayurvedic medicine contains Hg, some recent in-vitro and in-vivo studies did not find any toxic effect of these Hg-based Ayurvedic remedies (Dwivedi et al., 2013; Kumar et al., 2006; Sathya et al., 2008; Thakur et al., 2014). Notably, there are no such reports in Ayurvedic texts showing the adverse effect of these Hg-based Ayurvedic medicines when appropriately prescribed. Therefore, the questions needed to be answered are; the Ayurvedic mercury-based drugs toxic or not? And why Ayurvedic made HgS is superior to commercial procured HgS in medicinal uses? The objective of this study is to find out the neurotoxicity of the Ayurvedic Rasasindura and Kajjali and compare it with the toxicity developed with $HgCl_2$ and commercial HgS standard on zebrafish.

Ayurvedic Kajjali and Rasasindura medicines are prepared from raw liquid Hg and sulfur with a tedious and lengthy Ayurvedic procedure including purification, mixing and heat treatment, unlike Chinese medicine, which uses cinnabar ore directly. Kajjali is an intermediate in the Rasasindura preparation procedure and exhibits β-HgS crystalline form (Thakur et al., 2014) which is also used as a medicine. The final product, Rasasindura, is a highly pure single crystalline α-HgS phase (Ramanan et al., 2015) with a trace amount of impurity. A fraction of particles of these Ayurvedic medicines measures in nano-size (< 100 nm), and exhibits the robust crystal structure (crystal defect < 3%) (Ramanan et al., 2015). Therefore, it may be assumed that the efficacy of these Hg-based medicines is driven by the novelty in the Ayurvedic preparation procedures. It is worth noting that there are no reports in the literature which evaluate the effects of Ayurvedic Kajjali and Rasasindura medicines when administered to zebrafish model.

In this study, zebrafish were treated with four different mercury-based inorganic compounds, two of a chemical origin namely $HgCl_2$ and HgS, and two of a medicinal origin, namely Rasasindura and Kajjali (mercury-based Ayurvedic herbo-mineral medicines). The change in the several behavioral parameters of zebrafish was analyzed. The whole body cortisol level was measured to analyze the physiological reaction to stress, which is a valuable parameter for understanding development of any stress due to the toxicity. The behavior of zebrafish was studied using the video-tracking method, which could unveil the neurotoxicological aspect of these medicines if present. idTracker, an open source MATLAB program reported in the literature (Pérez-Escudero et al., 2014), was used for the video-tracking purpose, which was followed by indigenously developed MATLAB code to quantify all behavioral parameters.

2. Materials and methods

2.1. Chemicals

$HgCl_2$ (purity ~ 98%), and HgS (α-HgS, cinnabar, purity ~ 99%) were purchased from SRL Pvt. Ltd., India, and Sigma-Aldrich, USA, respectively. HNO_3 for ICP analysis was procured from Merck, Germany. Rasasindura and Kajjali were generously gifted by Shree Dhootapapeshwar Pvt. Ltd., India. The cortisol ELISA kit was procured from DBC Inc., Canada.

2.2. Zebrafish housing

In-house zebrafish facility was developed. Adult wild-type (long-fin, 12–15 months old) zebrafish, procured from a local fish supplier, were used for this behavioral study. The zebrafish were habituated in the laboratory in the recirculating water system maintained at 25 ± 1 °C for three months before the start of the study. The re-circulating water system contained both mechanical and biological filtration units to maintain the water quality (pH, hardness, nitrogenous wastes) suitable for zebrafish (Lawrence, 2007; Westerfield, 2000). For the zebrafish study, CPCSEA guidelines, Government of India, were followed.

2.3. Experimental design

For the experiment, 70 adult fish were chosen according to their size and weight (0.5–0.8 g) and divided into five groups (n = 14). The gender and body weight distribution of fish in every group were same. The fish of group-1 were used as vehicle control (VC), and those of group-2, group-3, group-4, and group-5 were induced with $HgCl_2$ (positive control, PC), HgS-standard (HGS), Rasasindura (R), and Kajjali (K), respectively. For each group, three number of tanks were allotted to carry out three independent experiments, designed for each group namely experiment-A, experiment-B, and experiment-C.

Experiment-A: (n = 6, male:female sex ratio = 2:1) The fish of experiment-A was used to quantify basic motor behavioral parameter study. The basic motor parameter was quantified for 5 days before the drug treatment and during the 5 days of drug treatment. After the completion of motor behavior experiment on the 5th day of drug treatment, the fish were euthanized, and whole body cortisol was quantified.

Experiment-B: (n = 4, male:female sex ratio = 1:1) The experiment-B was conducted to analyze the bioaccumulation of Hg in zebrafish tissue after the completion of 5 days drug treatment.

Experiment-C: (n = 4, all female fish) Color preference test and diving phenotype test were conducted on the experiment-C fish. The color preference test was carried out on the 5th day onward (on 5th, 6th and 7th day) after completion of drug treatment. The diving behavior test was conducted on the 28th day after drug treatment.

2.4. Dose preparation

The doses of R, K, and HGS were given orally mixed with dry granular food. The dose preparation was a critical step as there was a chance of elution of submicron drug particles in the water. To overcome this problem, 5 mg Rasasindura/Kajjali/HgS was mixed in an agate mortar with 50 μL cod liver oil (Seven Seas, Merck India). Next, 0.2 g dry food grain was added and mixed thoroughly for 15 min to attain the homogeneity. The insoluble HgS particles stuck with dry food grain properly due to the addition of cod liver oil. The drug mixed dry food was divided into twenty doses containing 0.01 g each (for six fish). Thus, theoretically, one drug dose (0.01 g) contain 0.00025 g (0.25 mg) of an individual drug, which was equivalent to 4 g drug dose/55 kg human body. The maximum limit of prescribed Ayurvedic drugs < 125 mg per day (Gokarn et al., 2012) for certain diseases.

2.5. Feeding and drug treatment

For the basic motor parameter measurements, the experiment-A tanks were initially used for video recording.

The fish of individual groups (see Table 1) were transferred to 2 L tank (dosing tank). Next, the drug dose or food (0.01 g) was given 30 min prior to video recording for experiment-A fish (Fig. 1). Video capturing was done after the drug dosing for individual groups separately from the experiment-A tanks (n = 6) after transferring the fish into the novel tank from dosing tank. Initially, for five days, tracking was done without any drug doses (data were represented as the control