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RESEARCH ARTICLE

Toxicity Analysis of Cardio Protective Homeopathy Drugs *Aconitum napellus* and *Digitalis purpurea* using Zebrafish Embryo

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ABSTRACT

Homeopathy practice is one of the age-old alternative medical practices followed worldwide. In this study, we determined the toxic potential of two homeopathy drugs namely *Aconitum napellus* and *Digitalis purpurea* using zebrafish embryos, which are used to treat cardiovascular disorders by homeopathic procedures in humans. Our study implies that both drugs are potentially toxic at lower dilution such as 10^{-1}, which even showed absolute mortality. Reduced heartrate and pericardial edema was observed at 10^{-2} dilutionin *A.napellus*, and at 10^{-2} and 10^{-3} dilutions in *D purpurea*. However, other developmental abnormalities, such as reduced heart rate, heart malformation, and hatching rate were not observed at higher dilutions ranging from 10^{-6} to 10^{-12}. Based on the results we conclude that higher dilutions of these two drugs could be used to investigate the cardioprotective role in zebrafish embryos. Hence, at higher dilutions, as given in homeopathic practices these drugs may have possible anti-cardiovascular effects.

Keywords : *Aconitum napellus*, *Digitalis purpurea,* pericardial edema, cardioprotective, zebrafish

INTRODUCTION

Homeopathy is an alternative system of medicine to treat a range of illness. It was developed by Samuel Hahnemann (1755–1843) from Germany almost 200 years ago [1]. The drugs used in homeopathy are obtained from the extracts of plants, animals, and mineral sources. In this method, drugs will be used to activate the body's self-healing power by

stimulating appropriate reactions in the body in response to the symptoms. Homeopathy is based on two fundamental principles: "Like cures like"- a substance that causes specific signs and symptoms in a healthy individual can be utilized as a medicine for patients who have comparable disease symptoms [2]."Law of minimum dose" – according to this law, the drug retains biological activity after repeated dilution beyond Avogadro's number, that is the highly potentized formulations give observables effects on healthy individuals, but with relatively higher doses it may aggravate the ailment by enhancing the parallel symptoms and adverse effects [3]. Globally, homeopathy is practiced in more than 85 countries. In India, the system was first practised in Bengal in the early 19th century and has spread across the country [4]. Now it is quite popular and has evolved as an alternative medical system in India. The homeopathy drug market is growing 25% annually and more than 100 million people are following this practice for various health-related complications including HIV, Asthma, skin diseases, cancer, heart diseases, and diabetes [5]. Moreover, administration of homeodrugs is simple, its accumulation is limited inside the body, and are environmentally sustainable [6].

Homeopathy is also well-known for its critics and controversies, which arise from skepticism about the drug's efficacy due to the lack of scientific evidences [7]. The main concern is that the homeopathic drugs are extremely diluted, so they may not contain a considerable quantity of active ingredients. It is also uncertain arguemented that the biological activity of these drugs is still being maintained at higher dilutions [8]. The presence of heavy metals, and toxic nature of drug source, may have negative consequences among some patients when administered with high concentrations of the drugs. Moreover lack of scientific evidence to establish the mechanism of homeopathy drugs limited their applications to treat diseases. In this study, we used two homeopathy drugs namely *Aconitum napellus* and *Digitalis purpurea*, which are already used as a remedy for cardiac and other common ailments. Both of these drugs are made from plant extracts which are highly toxic by nature [9].A study with alkaloids extracted from the Aconite plant shows that it induces cardiotoxicity along with yolk sac edema in zebrafish embryos at the concentration of 2.5µg/L [10]. However, *Aconitum napellus* and *Digitalis purpurea* are used to treat heart related problems in homeopathy and other traditional medicines [11]. In fact, *D. purpurea* has been used as a drug for emetics and heart diseases among Egyptians and Romans from ancient time to till date [12].

In the present study, we used zebrafish embryo as a model to evaluate the toxicity induced by the above mentioned homeopathy drugs. Zebrafish is an excellent model organism for toxicological studies and is used widely to elucidate the underlying molecular mechanisms of toxicity and to predict risk on humans and for preclinical drug discovery and screening [13,14]. To study the biological activity of the drugs, it is important to determine the non-lethal concentrations of these two drugs. Therefore the non-lethal concentrations were determined prior to study the efficacy of the drugs for the treatment of treat heart diseases. To determine the non-lethal concentrations of *A.napellus* and *D. purpurea,* we analysed the parameters such as percentage of mortality, hatching rate, heart rate, and teratogenic potential with different dilutions

MATERIALS AND METHODS

Zebrafish Maintenance

Adult wild-type Zebrafish were purchased from a local aquarium and carefully transferred to the laboratory in oxygenated polythene bags. In the laboratory, the fishes were maintained in glass tanks (35 X 17 X 18 cm LBH). The fishes were acclimatized for two weeks. The male and female fishes were then kept in separate tanks with adequate aeration. The room temperature was maintained at 26 ± 1°C and 14:10 h light-dark cycle was provided. The fishes were fed with dry floating pellet feed (Toyo brand red and green floating pellet) and frozen brine shrimp (*Artemia*). Tank water was changed once in two days at the regular time point and water quality was checked regularly.

Breeding and Collection of Embryo

Male and female fishes were placed in breeding tanks with an equal ratio and kept in the dark to induce spawning. The fish were exposed to light for 40 minutes the next morning after the barrier was removed. The fertilized embryos

were collected with a filter and placed in a petri dish containing E3 medium. The collected embryos were washed twice with E3 medium and unfertilized and undeveloped embryos were removed with pasture pipette by observing under a stereomicroscope (NIKON stereo microscope).

Drug Dilutions and *In vitro* Embryo Toxicity Assay

The embryotoxicity test was carried out according to the OECD guideline 236 [15]. The mother tincture of the drugs *Aconitum napellus* and *Digitalis purpurea* were purchased from Bhandari Homeopathic laboratories, Faridabad, India. The mother tincture was briskly shaken and diluted serially in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM, $MgSO_4$) on X scale (Homeopathy decimal scale) ranged from 10^{-1} to 10^{-12}. Embryos with the four-cell stage (n=20/dilution) were chosen and exposed to diluted drugs for 96h in 12 well plates with 3ml of drug solution from appropriate dilution. E3 medium without drugs was used as control. Plates were incubated at 26 ± 1ºC with a 14:10 LD cycle. The solutions were changed every day until the end of the experiment.

Mortality and Hatching Rate of Treated Embryos

The number of dead embryos was recorded and removed once in every 24hours. The percentage of mortality for different dilutions of the drug was computed based on the number of dead embryos at each time interval. The hatching rate was calculated at the following three different time points: 48hpf, 72hpf, and 96hpf.

Analysis of Heartbeat Rate

The heartbeat rate was counted manually in both control and drug-treated groups under a stereomicroscope at 96hpf. Embryos were immobilized using 3% methylcellulose in a glass slide and positioned using an embryo loop. The number of heartbeat per 30sec was calculated.

Analysis of Developmental Abnormalities

The developmental stages were monitored every 24h, until 96hpf under a stereomicroscope (NIKON Eclipse), and compared to the stages described by Kimmel *et al* [16]. The developmental stages were documented pictorially and analyzed for possible abnormalities.

Statistical Analysis

All experiments were carried out in triplicates. The mortality and hatching rate was calculated and represented in percentage. The heartbeat rate of embryos with various concentrations (10^{-1} to 10^{-12}) of homeopathy drugs was analyzed by one-way ANOVA with Dunn's multiple comparisons. Statistical significance was accepted at $p<0.05$. All statistical analyses were performed using GraphPad Prism 6 (Version 6.01).

RESULT

Mortality

Mortality was recorded in every 24h and the cumulativepercentage of mortality was determined after 96hpf.The result showed that 100% of mortality was observed in 10^{-1} dilution in both drugs and both resulted in immediate mortality. In addition, *Digitalis purpurea* (DP) resulted in 100% mortality with 10^{-2} and10^{-3} dilutionsat 6hpf and 96hpf respectively (Fig 1).

Hatching Rate

In zebrafish, hatching starts from 48hrs and maximum hatching occurs in 72hpf [16]. In this study, the hatching rate of embryos was noted from 48hpf to 96hpf. It was found to be 100% at 96hpf with 10^{-5} to 10^{-12} dilutions. Comparison between concentrations of drugs and hatching rate didn't show any significant variation in both drugs. However, the percentage of hatching rate was more than 50% with *A. napellus* at 48hpf. In case of *D. purpurea,* observed hatching rate was less than 50% at 48hpf (Fig.2).

Heartbeat Rate

Heartbeat rate was monitored at 96hpf. Compared with control group, A. *napellus* treated groups showed significantly reduced heart rate in 10^{-2} dilution (Fig.3.a) whereas with *Digitalis purpureait was observed* in 10^{-4} dilution (Fig.3.b). However, no significant variation was observed with remaining dilutions of both drugs.

Developmental Deformities

Developmental deformities such as pericardial edema, tail and jaw deformation, and cornea development were observed at lower dilutions. Among them, pericardial edema was predominatly observed on embryos treated with *A. napellus* at 10^{-2}dilution and at 10^{-2} and10^{-3} dilutions with *Digitalis purpurea* (Fig 4 A and B.). With lower dilutions, no significant malformations were observed.

DISCUSSION

The toxicity testing of pharmacological products gives a better understanding about the non-lethal concentration of substances and also gives the conceivable toxic effects of those products [17]. In the present study, the toxicity potential of homeopathy drugs *A.napellus* and *D. purpurea* was investigated using zebrafish embryos, which is the best model for screening toxicity potential for drugs and toxic compounds [18]. Though the drug is used to treat cardiac ailment, the toxicity levels should be standardized for different model organism because the toxic level and efficacy of the drug depends on species, size and age of the testing organisms [19]. Identifying effective concentrations allows researchers to investigate the molecular aspects of the drug and as well as to treat the heart diseases in the model organism.

In this study, *A. napellus (AN)* caused 100% mortality athigher concentration (10^{-1}), whereas treatment with*D. purpurea* (DP) showed 100% mortality with 10^{-1}, $10^{-2}$and 10^{-3}dilutions in zebrafish embryos. Aconite present in the *A. napellus* interacts with the voltage-dependent sodium channel present on cell membranes of excitable tissues, including myocardium, striated and smooth muscle, and neurons, altering membrane depolarization and repolarization. It showed high affinity to the voltage-sensitive sodium channel in its open state and inhibit the conformational change to the inactive state [20]. It delays repolarization by prolonging sodium influx and membrane depolarization[21]. Moreover, aconite increases the strength of muscle fibre contraction by increasing acetylcholine release from nerve endings at lower concentrations. At higher concentrations, aconite depress the muscle contraction by maintain the sodium channels in its open state and reduced the release of acetylcholine at axonal end [20, 22].Recently the cardio protective role of aconite was studied in rats and implies that aconite enhances the heart function by exerting anti myocardial ischemia effect through the PI3K/Akt signalling pathway and also reducing myocardial fibrosis, inflammatory responses [23]. In the present study, as expected reduced heart rate was observed with lower dilutions (10^{-2} to 10^{-5}) and normal heartbeat rate was observed with higher dilutions.

D. purpurea contains a cardiac glycoside, digoxin which has the potential to modulates the heart functions and acts as an anti-inflammatory, anti-oxidant, anti-tumor, and hepatoprotective agent [24,25]. Digoxin inhibit the Na+ /K+ -ATPase pump by binding the K+ binding site in the myocardium, which results in increase in the intracellular sodium concentration at the same time decrease in potassium concentration. This elevated sodium level results in increased intracellular calcium level, which raises the action potential of cardiac cells [26].

Aconite and Digitalis have long been explored for their cardio protective properties [27]. Antioxidant and anti-inflammatory potential of these drugs are important in preventing cardiovascular diseases [24,25,28]. Meanwhile, a case study reveals that both the drug induces cardiotoxicity when ingested at higher concentration; in both cases patients took the medicine without proper advice from the physicians [29, 30]. In the present study lower dilutions of drugs reduce the heart beat rate, whereas normal heart rate was not affected with higher dilutions of the drugs. Among the developmental deformities, we found that *A. napellus (AN), and D. purpurea* (DP) resulted in 100% pericardial edema at 10^{-2} and 10^{-2} and 10^{-3} dilutions respectively. As a response to cardiotoxicity induced by the drug

at higher concentrations, pericardial edema, reduced heart rate, and high mortality were observed. In addition, cardioavascular functions were not affected at lower concentrations of the drugs. It is clear from the above study that lower dilutions of the both drugs causes morphological and physiological changes in the heart, but in higher dilutions, no such effects were observed.

CONCLUSION

Homeopathy drugs are prepared from material which is highly toxic by nature. From this study, we conclude that higher dilutions' ranging from 10^{-6} to 10^{-12} doesn't show any significant effects on developing zebrafish embryos. Hence, higher dilutions of *A. napellus (AN),* and *D. purpurea* (DP) might be utilised to investigate the cardio protective roles against the cardiac problem induced by toxic substances using zebrafish embryos..

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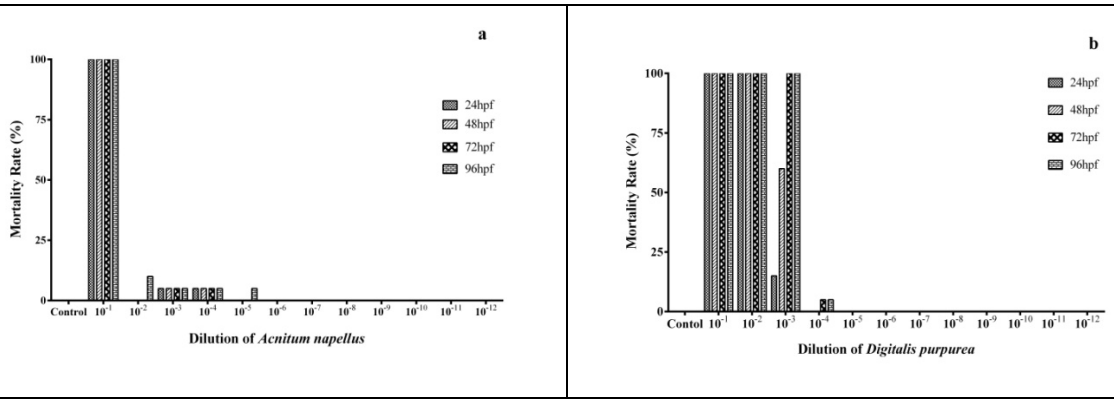

Fig.1. Mortality rate of zebrafish embryos treated with homeopathy drugs (a) *Aconitum napellus* (AN) and (b) *Digitalis purpurea* (DP)after 96 hpf.

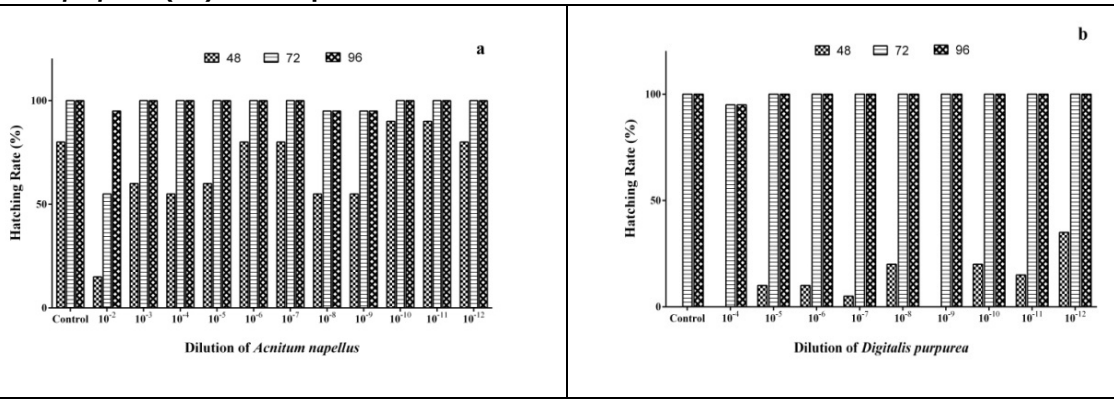

Fig.2. Hatching rate of zebrafish embryos treated with homeopathy drugs (a) *Aconitum napellus* (AN) and (b) *Digitalis purpurea* (DP)after 96 hpf.

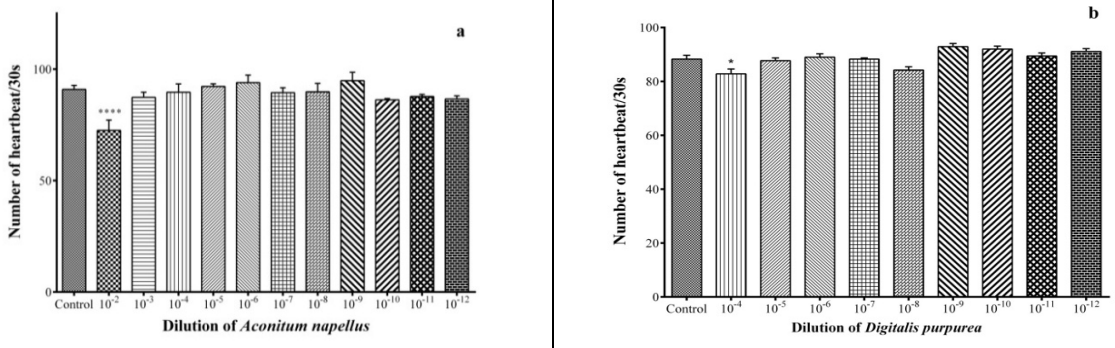

Fig. 3. Heartbeat rate of embryos treated with homeopathy drugs after 96 hpf(a) *Aconitum napellus* (AN), and (b) *Digitalis purpurea* (DP). Data are expressed as the mean ± SEM and asterisks (*) indicate statistically significant differences between groups, *p<0.05; **p<0.01; ***p<0.001; ****p<0.001 (One-way ANOVA with Dunn's multiple comparison).

Fig. 4. Developmental and malformations of zebrafish embryos treated with (A) *Aconitum napellus* (AN), and (B) *Digitalis purpurea* (DP). Number represents the concentration of used drugs. Pericardial edema (PE) was observed with the lower dilutions of both drugs.