Evaluation of the Toxic Effects of Clozapine in Zebra fish (Danio rerio) embryos with the Fish Embryo Toxicity Test

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ABSTRACT

Clozapine (an atypical antipsychotic drug) is indicated in treatment-refractory schizophrenia. Antipsychotics have been reported to have cardiotoxic effects in humans. The zebra fish (Danio rerio) is a tropical cyprinid used in toxicological research. In the present study, the toxicity of clozapine was evaluated in zebra fish embryos with the aid of the fish embryo toxicity test (FET). The endpoints monitored included circulation, heart rate, pericardial oedema, tail extension, spinal deformation, hatching time, coagulation and death. Clozapine was tested at five different concentrations ranging from 1 µg/L to 10 mg/L. The toxic effects observed were a marked bradycardia at 10 mg/L and various abnormalities in the zebra fish embryos exposed to clozapine at 1 µg/L to 10 mg/L. Based on the results obtained in this study, clozapine is toxic to zebra fish embryos and its level in the aquatic environment should be closely monitored in order to forestall adverse effects on the reproductive capabilities and survival of aquatic organisms.

Keywords: Abnormalities, Bradycardia, Clozapine, Endpoints, Fish Embryo toxicity Test, Zebra fish

Introduction

Clozapine is an atypical antipsychotic and a tricyclic dibenzodiazepine derivative [1]. It appears to be the most effective antipsychotic for treatment-resistant schizophrenia but its use is limited because of the risk of agranulocytosis, myocarditis, sedation, convulsion, obesity and diabetes mellitus [2]. The administration of clozapine to patients is associated with fewer incidences of extrapyramidal effects [3]. Clozapine therapy is associated with improved efficacy in the management of positive and negative symptoms, cognitive function and well being of patients [4]. Clozapine is a polyreceptor antagonist with affinities for serotonin, dopamine, muscarinic and histamine H1 receptors [5]. It undergoes rapid absorption and extensive metabolism following oral administration and it has a half life of about 6 hours [6, 7]. In addition, it has a bioavailability between 27% and 50% when administered orally [8]. The antipsychotic, clozapine, undergoes extensive metabolism in humans and its two main metabolites are N-desmethylclozapine (pharmacologically active form) and clozapine –N- oxide [9]. It is similar to benzodiazepines with regard to pharmacological and behavioural features [10]. However, antipsychotic therapy in humans is associated with prolonged QT interval, culminating in torsades de pointes and sudden death [11]. The prolongation of the QT interval is attributed to the blockage of Human ether-a-go-go-related gene (HERG) potassium channel by antipsychotics, particularly clozapine [12]. Furthermore, there is sparse information on the effects of clozapine on the developmental stages of humans and animals. However, reproduction studies in rats and rabbits at doses two to four times the human dose did not elicit infertility or fetal abnormalities [13]. But in an investigation conducted by [14], clozapine decreased fetal heart rate variability in a human infant. Breast feeding has been identified as a route of entry of clozapine into the fetal circulation [14]. This can evoke sedation and muscle flaccidity in the neonate [15]. The zebra fish is a tropical cypriniform (Family: Cyprinidae) and its name is derived from its striped integument. It is characterized by external development, transparency during organogenesis and a tractable diploid genome which makes it a suitable model organism for research [16]. It is a valuable organism for the initial stages of drug
discovery [17]. Zebra fish embryos are permeable to drug molecules in suitable media and hence their use for drug toxicity testing. Also, zebra fish are currently being used for teratogenicity assays for evaluating the teratogenic potential of chemicals [18]. It has a small size and this is advantageous in terms of holding space, husbandry costs and drug doses [19]. Also, whole-body sectioning and histological evaluation of the zebra fish is feasible for research purposes.

Residues of clozapine have been detected in sludge from Swedish sewage effluents [20] but there is limited aquatic ecotoxicity data on clozapine. Therefore this study was conducted in order to investigate the toxicity of clozapine to aquatic organisms with the use of zebra fish (*Danio rerio*) embryos as model organisms. The fish embryo toxicity test [21] was employed in this study.

Materials and Methods

Experimental animals

Adult zebra fish (*Danio rerio*) were procured from a local supplier in Uppsala, Sweden and were allowed to adapt to laboratory conditions for 4 weeks before the commencement of the project. The study was conducted at the Division of Pathology, Pharmacology and Toxicology, Swedish University of Agricultural Sciences, Uppsala, Sweden.

The animals were kept in aquaria within a temperated laboratory at 26 ± 1°C and a 12 – h light /dark cycle was maintained. Standardized water (ISO 7346 – 1, 1996) was used for the maintenance of the adult zebra fish. It was prepared from deionised water and the following salts were added: CaCl$_2$ x 2H$_2$O (117.6 mg/L), MgSO$_4$ x 7H$_2$O (49.3mg/L), NaHCO$_3$ (25.9mg/L) and KCl (2.3 mg/L) (Sigma Aldrich Sweden AB) to produce standardized water. The adult zebra fish were fed with newly hatched *Artemia nauplii* (San Francisco Bay brand) 2 times daily.

The animals were cared for in accordance with the guide to the care and use of experimental animals. The use of the animals was reviewed and approved by the Swedish University of Agricultural Sciences Animal Care Review Committee.

The Fish Embryo toxicity Test (FET)

The eggs were obtained from adult male and female zebra fish placed in cone shaped breeding funnels for spawning in a ratio of 2:1. The funnels were filled with 20 – 25 liters of standardized water. The eggs were obtained from the breeding funnels the following day between 30 and 60 minutes after light was switched on in the laboratory. The brood fish were thereafter returned to the aquaria.

Stock solutions of clozapine (Sigma Aldrich Sweden AB) were prepared in dimethylsulphoxide (>99.5% DMSO; Merck) and then diluted 1:1000 in standardized water to exposure concentrations of 1 µg/L, 10 µg/L, 100 µg/L, 1 mg/L and 10 mg/L. Standardized water and DMSO were used as negative controls. The concentration of the carrier DMSO was 0.1% in the final test and negative control solutions. A mixture of musk ketone 50 µg/L (Sigma Aldrich Sweden AB) and 7.6 mg/L phenylthiourea (Sigma Aldrich Sweden AB) was used as positive control for bradycardia and inhibition of pigmentation respectively. Newly laid eggs were placed in Petri dishes containing the test solutions with the aid of a pipette.

The eggs were examined in a stereo microscope for the selection of eggs that had reached the four – cell stage. For each exposure concentration, 12 eggs were selected and transferred individually to 96 micro – well plates (Costar, Corning Incorporated, USA) with 250 µL of the test solutions. The plates were covered with parafilm and then kept at 26 °C in a 12 – h light/dark cycle. The examination of the eggs was carried out with the aid of a stereo microscope at 24, 48 and 144 hours post fertilization (hpf). In order to prevent thermal stress to the embryos, microscopic examination was performed with a light source equipped with a fiber optic cable. Photos of the micro – well plates were taken with a mounted digital interval timer camera (Canon Power Shot Pro I) once every hour from 48 to 144 hours.

The various endpoints monitored included tail extension (24h), spontaneous movement (24h), pigmentation (24h), eye development (24 and 48h), coagulation (24 and 48h), heart rate (48h), pericardial oedema (48h), circulation (48h), spinal deformation (144h) and hatching time (144h). The tail extension of the zebra fish embryos was measured on a scale of one to three, where one denoted the complete detachment of the tail from the yolk sac, two
denoted partial extension while three denoted lack of detachment of the tail from the yolk sac. The heart rate was determined by observing the time taken to count 30 heartbeats. The circulation was observed as the flow of blood in the caudal artery. The spinal deformation of the zebra fish embryos was measured on a scale of one to four, where one denoted the absence of deformation, two and three denoted curvature at less than 45° and greater than 45° respectively while four denoted curvature at several locations. The pigmentation of the embryos was measured on a scale of one to four, where one denoted full pigmentation, two and three denoted a reduction in the intensity of pigmentation, while four denoted the absence of pigmentation. Circulation, eye development and oedema were measured as categorical data (yes or no basis).

In the fish embryo toxicity test, the lethal endpoints include coagulation of egg, non detachment of the tail from the yolk, lack of somites and absence of heart beats. The completion of gastrulation, eye development, spontaneous movement, circulation, pigmentation, oedema and heart rate are regarded as sub-lethal endpoints that could be measured in order to determine the mode of action of the toxic response.

The study was terminated by euthanizing the zebra fish in tricaine methane sulphonate (MS – 222, Apoteket AB, Sweden).

**STATISTICAL ANALYSIS**

The data were analyzed by one-way ANOVA followed by Dunnett’s post-hoc test for comparison of exposed groups with controls (DMSO carrier). However, categorical data such as coagulation, circulation and eye formation were analyzed with the Chi Square test. The software used for analyzing the data were Statview 5.0.1 (SAS Institute Inc.) and MINITAB release 14 (Minitab Inc.). The level of significance was set at 0.95 (p < 0.05). Data was presented as mean ± standard deviation (SD) unless otherwise stated.

**RESULTS**

Bradycardia and reduced viability were elicited in the zebra fish embryos exposed to clozapine at 10 mg/L (Figure 1). The hatching time was prolonged in zebra fish embryos exposed to clozapine at 100 µg/L but this finding was not significant. The zebra fish embryos exposed to clozapine at 10 mg/L failed to hatch (Table 1). Several abnormalities such as coagulation, eye defects, pericardial oedema, absence of circulation and malformations were observed in zebra fish embryos exposed to all the concentrations of clozapine (1 µg/L – 10 mg/L) (Table 2).

<table>
<thead>
<tr>
<th>Doses</th>
<th>Hatching Time (Mean ± Standard Deviation)</th>
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<tbody>
<tr>
<td>1 µg/L</td>
<td>50.4 ± 2.9</td>
</tr>
<tr>
<td>10 µg/L</td>
<td>52 ± 4.3</td>
</tr>
<tr>
<td>100 µg/L</td>
<td>55.5 ± 4.9</td>
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<tr>
<td>1 mg/L</td>
<td>52.8 ± 3.9</td>
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<tr>
<td>10 mg/L</td>
<td>0</td>
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<tr>
<td>Control (Standardized water)</td>
<td>51.9 ± 3.4</td>
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<tr>
<td>Control (Dimethyl sulphoxide)</td>
<td>49.9 ± 2.3</td>
</tr>
</tbody>
</table>

Table 1. Hatching time (hours) of zebra fish embryos exposed to clozapine (1 µg/L – 10 mg/L).
Table 2. Abnormalities observed after exposure of zebra fish embryos to clozapine (1 µg/L – 10 mg/L).

<table>
<thead>
<tr>
<th>Doses</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
<th>f</th>
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</thead>
<tbody>
<tr>
<td>1 µg/L</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>10 µg/L</td>
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<tr>
<td>100 µg/L</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>1 mg/L</td>
<td>1</td>
<td>-</td>
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<td>-</td>
<td>1</td>
</tr>
<tr>
<td>10 mg/L</td>
<td>-</td>
<td>5</td>
<td>4</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Water</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>DMSO</td>
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<td>-</td>
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<td>-</td>
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<td>-</td>
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<td>1</td>
</tr>
</tbody>
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a (coagulation), b (eye defects), c (oedema), d (no circulation), e (unhatched), f (malformation), g (tail deformation) and h (death)

DISCUSSION

Antipsychotics that cause QT prolongation in humans have also been reported to cause bradycardia and atrioventricular block in the zebra fish [10]. This is in accordance with the bradycardia elicited by clozapine in this study. In an investigation conducted by [10], zebra fish embryos (2 days post fertilization) were exposed to various concentrations of cardiotoxic drugs including clozapine for 4 hours. Clozapine elicited bradycardia, pericardial oedema, enlarged heart chambers and slower circulation in the zebra fish. This corroborates the findings in our study.

Furthermore, clozapine evokes cardiotoxicity in humans by receptor blockade, disruption of the cardiac conduction system, prolonged QT interval, left ventricular dysfunction, sino-atrial node abnormalities, myocarditis, postural hypotension, polydipsia-hyponatremia syndrome, weight gain and glucose intolerance [11]. It has been observed...
that the effects of cardiotoxic drugs such as clozapine in zebra fish are similar to those in primates and other animal models [12].

In this study, clozapine elicited various abnormalities in zebra fish embryos including circulation defects, gross morphological defects and pericardial oedema. This suggests that clozapine might have teratogenic effects in these organisms. However, there is limited literature on the effects of clozapine on fetal and neonatal development in humans and animals. However, atypical antipsychotics such as clozapine do not seem to be associated with an increased risk for teratogeny in pregnant women [13]. It is likely that zebra fish (Danio rerio) embryos are susceptible to the teratogenic effects of clozapine based on the results obtained in this study.

However, clozapine residues have been detected in sludge from Swedish sewage effluents [8] and their effects on aquatic organisms is unknown. In this study, zebra fish was used as a model for aquatic organisms and clozapine elicited toxic effects in zebra fish embryos.

CONCLUSION AND RECOMMENDATION

Clozapine is toxic to zebra fish (Danio rerio) embryos. Its residues should be closely monitored in the aquatic environment to forestall adverse effects in aquatic organisms, especially fish. However, fish are important sources of animal proteins and omega-3 fatty acids and there are concerted global efforts aimed at boosting fish production for human and animal consumption. Therefore it is crucial to investigate the effects of drug residues in the aquatic environment in order to detect their potential reproductive and developmental toxicities that could be detrimental to the survival and propagation of aquatic organisms including fish.

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REFERENCES