Exposure to 7, 8 Dihydroxycoumarin protects Cadmium induced toxicity effect in Zebrafish (Daniorerio) embryos

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Abstract

Cadmium (Cd) is one of the precedence pollutants in the environment which menaces the aquatic organisms. Cd is also shown to have deleterious health impairments causing male and female infertility in humans. In this study, we investigated the role of 7, 8-DHC as a potent rescuer of Cd induced toxicity using zebrafish (Daniorerio) model. The anti-oxidant property of 7,8-DHC was analysed and confirmed using 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric ion reducing power (FRAP) and hydroxyl radical (OH) assays.

Further, we induced Cd toxicity in zebrafish embryos at 100-µM concentration and the intoxicated embryos showed a significantly reduced survival, delayed hatching and phenotypic aberrations at 24, 48, 72 and 96 hours post fertilization (hpf). Similarly, Cd intoxicated embryos showed an expressively increased cardiac function (170 ±1 beats/min and 172± 1 beats/min) at 48 and 60 hpf. Furthermore, the rescuing effect of 7,8-DHC was analysed via treatment of Cd intoxicated embryos at dosage dependent manner. At 100 µg, 7,8-DHC showed significantly reduced heart beat compared to the Cd intoxicated embryos. Moreover, 50 and 100 µg of 7, 8-DHC treated groups showed 103± 1 beats/min and 104±1 beats/min respectively. Histopathological interpretations revealed the rescuing effects on notochordal segmentation at 50 and 100 µg 7, 8-DHC in Cd intoxicated embryos at 96 hpf. Overall, our results clinched that 7, 8-DHC could act as a potent redox scavenger against Cd toxicity.

Keywords: Cadmium, 7,8 –DHC, Treatment, Toxicity, Zebrafish Embryo.

Introduction

Pollution due to heavy metal causes severe deterioration of various biological processes in numerous life forms. Particularly, water pollution leads to accumulation of heavy metals in fish and thus hampering its breeding and development[17]. Cadmium (Cd) is a non-essential environmental toxicant heavy metal, earlier known to have no beneficial role in the human body. However, recent studies revealed that its exposure at even low concentration is reported to have severe health impairments such as adversely reduced fertility effect in humans and increased miscarriage during pregnancy etc[7].Globally, this potential toxicant was released into the environment by natural or anthropogenic activities such as mining, refining and the manufacturing and application of phosphate fertilizers[16].

Thus, the accumulation of Cd into the biomass of the organisms dwelling in the environment causing deleterious effect on food chain and food web finally leads to the loss of global biodiversity. The alternate potent scavenging mechanism to rescue the potential development of toxicity incurred due to intoxication of Cd is still unknown.

Coumarins are the anti-oxidants found naturally in various plant species such as green beans, lavender, apricots, strawberries, cherries and aloe and in lower organisms bacteria and fungi[22]. The polyphenolic secondary derivative of plants is reported to exhibit anti-inflammatory, antimicrobial, antivirus, anticancer, anticoagulant and antioxidant activities[3,6,26,32]. 7,8-Dihydroxycoumarin (Daphnetin) as represented in fig. 1 is one of the coumarin derivatives, is an active compound and also a potent anti-cancer, antioxidant, anti-inflammatory, anti-hypoxic, neuroprotective, anti-proliferative, anti-diarrheal and anti-parasitic compound[31,34]. Daphnetin is shown to have inhibitory effect on kinase activity *in vitro* and to exhibit significant free radical scavenging activity and inhibitory effects on lipid peroxidation[12,37].

Wang et al[33] reported that daphnetin inhibited the proliferation of A549 human lung adenocarcinoma cells, induced apoptosis via Akt/NF-κB signaling suppression. Additionally, it has been clinically used in the treatment for coagulation disorders and lumbago rheumatoid arthritis and anti-pyrogenic agent[13,36,38]. In the present study, we indented to analyse the anti-oxidant mediated rescue property of Daphnetin using Cd-intoxication using mammalian model system.

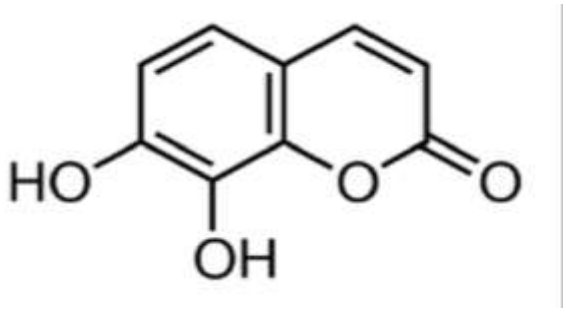

Fig. 1: Structure 7, 8-Dihydroxycoumarin

Zebrafish (*Daniorerio)* is a higher hierarchal vertebrate model system to understand the developmental process and provide more information prevailing on the events that occur during its embryonic development[27]. Due to the diminutive availability of data about the developmental deformities occurring after exposure to Cd[9], we designed our study to underscore the pathophysiological complications of Cd toxicity using this well-known vertebrate developmental model.

Cd intoxication is reported to cause nephrotoxicity, immunotoxicity, osteotoxicity and tumors after its prolonged exposures in humans[14,25]. A well-studied phenomenon is the formation and release of reactive oxygen species (ROS) often implicated in Cd toxicology[24,20]. Further, the released ROS triggers superoxide anion, hydrogen peroxide and hydroxyl radicals *in vivo* as detected by the electron spin resonance spectra. This in turn favours the activation of redox sensitive transcription factors (e.g. NF-κB, AP-1 and Nrf2) and alteration of ROS-related gene expression. Adaptive mechanism such as expression of metalloproteins, metallothionein and glutathione, evolved which in turn quenches the Cd-induced oxidative stress.

However, the survived intoxicated cells rendered apoptotic tolerance which in turn possess the inherent oxidative DNA lesions, potentially due to Cd intoxication and leading to tumorigenesis. Therefore, ROS elevation is the immediate effect of acute Cd toxicity, we also tried to characterize the ROS releasing potential of Cd using colorimetric assays in our current study. Our study hypothesized that the production of ROS may also be associated with Cd toxicity using Zebrafish model.

Further, these ROS may be scavenged by antioxidant defense systems and thereby rescue from Cd-intoxication event. In the current study, we assessed the antioxidant nature of 7,8-DHC followed by its rescuing potential in Cd intoxicated Zebrafish embryos.

Material and Methods

Chemicals and Reagents: The chemical compounds 7,8-Dihydroxycoumarin (7,8-DHC) (98% purity), 1,1-diphenyl-2-picrylhydrazyl DPPH and acridine orange (AO) were purchased from Sigma Aldrich Chemicals (St Louis, MO, USA) and all other chemicals were purchased from Merck India Pvt. Ltd.

In vitro antioxidant assays

DPPH radical scavenging Assay: The DPPH radical scavenging activity was assayed as described[21]. Briefly, the reaction mixture containing methanol dissolved DPPH and different concentrations of 7,8-DHC (2, 4,6,8 µg/ml) were incubated in dark condition for 30 min at 37ºC. After incubation, the reaction mixture was measured at 517nm in UV-Spectrophotometer (SHIMADZU). The quercetin was used as reference standard. The scavenging of DPPH was calculated as DPPH % of inhibition = [(ABS control- ABS sample) / (ABS control)] x100.

Ferric reducing antioxidant property assay (FRAP): The FRAP assay was performed according to the method[4]. The reaction tube containing 1.0 % potassium ferric cyanide and different concentration of 7,8-DHC (2, 4, 6, 8 µg/ml) in 0.2 M phosphate buffer (pH - 6.6) was prepared. The mixtures were kept incubated at 50 ºC for 20 min. Further after incubation, 10 % TCA was added to stop the reaction and the contents were centrifuged at 1000 rpm for10 min. Then the supernatant was collected and equal volume of distilled water and 1 ml of ferric chloride was added. The absorbance was recorded at 700 nm in UV Spectrophotometer (SHIMADZU). The quercetin was used as standard. The consumption intensity determines the antioxidant activity of 7,8-DHC.

Hydrogen peroxide assay (H_2O_2): The H_2O_2 assay was carried out following the procedure[28].Hydrogen peroxide (20mM) was prepared in 1.0 M phosphate buffer (pH 7.4). 7,8-DHC at different concentrations (2, 4, 6, 8 µg/ml) was added to hydrogen peroxide solution (1.0 ml). The mixtures were kept in incubation for 10 min at 37ºC. The absorbance was measured at 230 nm in UV- Spectrophotometer (Shimadzu). The blank solution contained the quercetin without H_2O_2. The percentage of H_2O_2 scavenging was calculated as:

H_2O_2 %inhibition = [(Abs control-Abs test)/ (Abs control)] ×100.

Maintenance of Zebrafish and embryo collection: Adult zebrafishes (both sex) were commercially purchased from local aquarium and maintained in a 50 L glass tank at temperature of 26±1°C according to described protocol with 14:10 hrs light/dark photo-cycle[40].The maintained zebrafish was fed with commercial spirulina micro pellets twice a day. Embryos were obtained from natural spanning of male and female (1:2 ratios) fishes overnight. The spawning was induced in the early morning by the onset of light source. Normally fertilized eggs who reached blastula stage were picked and maintained in E3 medium.

Experimental schedule: Zebrafish embryos at 5 hpf (hours post fertilization) were randomly sorted into six groups (n=15) in 6 well culture plates. Embryos maintained in E3 medium alone served as control. Group 2 was served alone only with 100 µM of Cd maintained in E3 medium.

Similarly, embryos in groups 3-6 were treated with 100 µM of Cd with different concentrations of treatment compound 7,8-DHC (1, 10, 50 and 100 µg/ml) in E3 medium for 96 hrs. The observation parameters including survival, hatching, heart rate and developmental changes were monitored at specific time intervals (12, 48, 72 and 96 hpf) under light microscope (MagnusMLXi, Olympus, Japan).

Developmental toxicity screening: The Cd exposure to the developmental toxicity profiles such as survival, embryo histopathological malformation, hatching and heart rate were monitored at 12 hrs time intervals for 96 hrs as described[18].Survival rate was calculated using the resting state of heart beat and the morphological deformities were studied using histopathological analysis. Further, the apoptotic lesions were studied using AO staining method using the successfully hatched out larvae in all the experimental groups of the embryos.

Apoptotic detection by acridine orange (AO) staining: Apoptosis persuading capacity of Cd and the apoptosis inhibitory effect of 7,8-DHC treated embryos were investigated by acridine orange staining as described[8]. After the exposure to Cd and treatment with 7,8-DHC at 96 hpf, the embryos were washed twice with embryo medium (E3) followed by exposure to 10µl acridine orange solution (5.0 µg/ml in E3 medium) for 20 min at room temperature. The embryos were washed with E3 medium and the apoptotic bodies were examined under a fluorescent microscope with an emission range of 525 nm (Magnus-MLXi, Olympus, Japan.)

Histopathological analysis: 96 hpf successfully hatched Zebrafish larvae were fixed with 10% formaldehyde and then embedded in paraffin wax. The zebrafish larvae sections (5.0 µm) were deparaffinised in xylene and rehydrated each slides with series (100, 70, 50, 30 and 10%) of ethanol at 10 min time interval. The slides were stained in hematoxylin for 5 minutes and washed in running tap water for 20 minutes. Further, the slides were counterstained with eosin for 15 seconds. After staining, the sections were dehydrated with graded ethanol, xylene cleared and mounted under the microscope using DPX mountant.The stained sections were observed under light microscope at x40 magnification (Olympus MLXi, Tokyo, Japan) and examined for histological alterations.

Statistical analysis: All experiments were conducted in triplicate and the data values were represented as mean ± SEM.The IC_{50} and hypothesis testing such as one way analysis of variance by ANOVA by Dunnett's test were performed to determine the statistically significant differences ($P<0.05$) between the means by using graph pad Prism, version 5.0 (San Diego, USA).

Results

Free radicals scavenging property of 7,8 –DHC: The compound 7,8-DHC showed dose-dependent (2, 4, 6, 8 µg/ml) increased inhibition of DPPH (Fig. 2a), FRAP (Fig. 2b) and H_2O_2 (Fig. 2c). It showed the free radicals scavenging potential of the compound with the IC_{50} range of 5.0, 2.29, 3.35 µg/ml respectively.

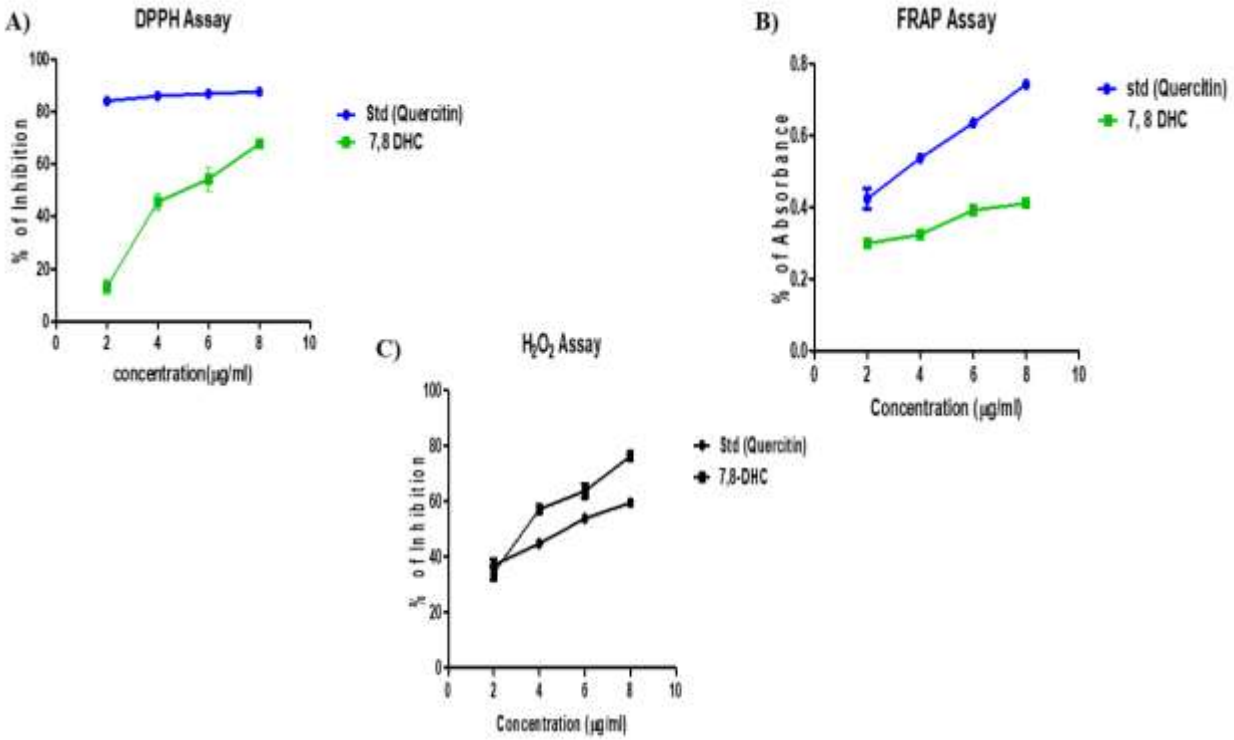

Fig 2: A) DPPH Free radical scavenging potential, B) Feric reducing antioxidant potential of Quercitin and C) The scavenging ability of Quercitin and 7, 8-DHC on hydrogen peroxide at different concentrations 7,8 DHC in different concentrations

Results partially suggested the radical donating potential of 7, 8-DHC *in vitro*. Based on these results, we suggest that the 7,8-DHC might act as an effective scavenger.

Effects of 7,8-DHC in survival, hatching and Heart beat rate on Cd intoxicated embryos: A significant reduction in the survival rate of the embryos approximately around 60% was observed in Cd intoxicated embryos as observed in the control Cd treated group (Fig. 3). However, co-treatment with different concentrations of 7,8-DHC (1, 10, 50 and 100 µg) significantly retained the survival rate to 50-90% and at concentration of 100 µg, 7,8-DHC showed maximum survival of zebrafish embryos when compared to 100 µM Cd at 96 hpf (Fig. 3).

Delayed hatching were observed in embryos exposed to Cd relative to the control group whereas the hatching rate increased under the presence of increasing concentrations of 7,8-DHC (Fig. 4). The overall inference of the assay is that the higher concentration of 7,8-DHC (100 µg) yields the higher hatching rate as observed as the control group. Only 54.3% of the Cd group came out of their chorines while the percentage of hatched embryos significantly increased to 72.9, 86.2 and 93.3 % for the 10, 50 and 100 µg 7,8 –DHC group respectively.

The heart rate regulatory effect of 7,8 –DHC on zebrafish embryos intoxicated with Cd at specific time interval 48 hpf and 60 hpf exhibited normal heart rate (122 ±1.2 beats/ min) without any lesions of abnormalities as in control. In contrast, Cd induced groups (100µM) exhibited significantly ($P<0.05$) increased heart rate (138 ±0.8 beats/min) along with a severe bradycardia noticed at 48 and 60 hpf when compared to control. However, 7,8-DHC (1, 10, 50, 100 µg) treated embryos showed significantly ($P<0.05$) decline in the heart rate (134 ± 1.5,68 ± 0.5,86 ±1.52 and 101 ±0.5 beats/min) respectively when compared to Cd group whereas 100 µg of 7,8– DHC treatment showed maximum normalizing effect on heart rate of zebrafish embryos at 60 hpf when compared with other doses, but not significant with each other. Hence, the above results depict the protective effects of 7,8 –DHC on Cd exposed zebrafish heart rate (Fig. 5).

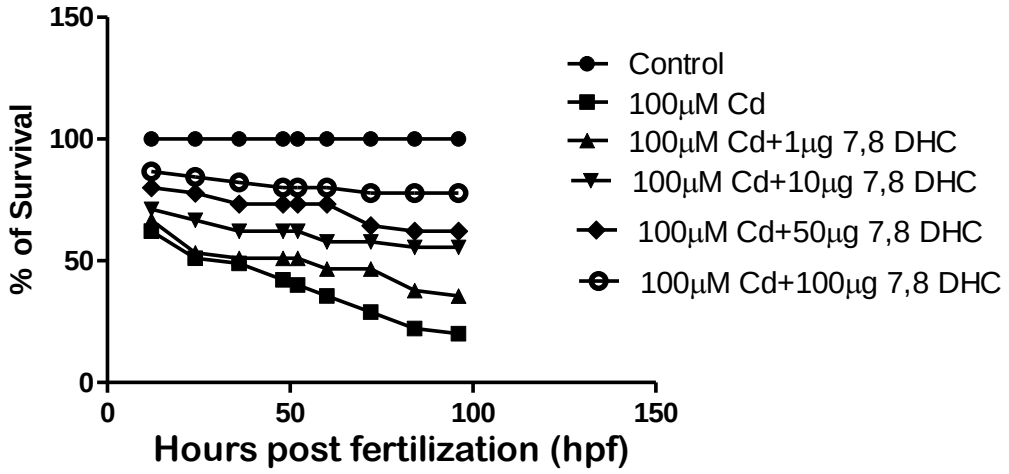

Fig. 3: Influence of 7, 8-DHC on survival of zebrafish embryos after exposed to Cd toxicant. Experiments were performed in triplicate and the values are represented as mean ±SEM.

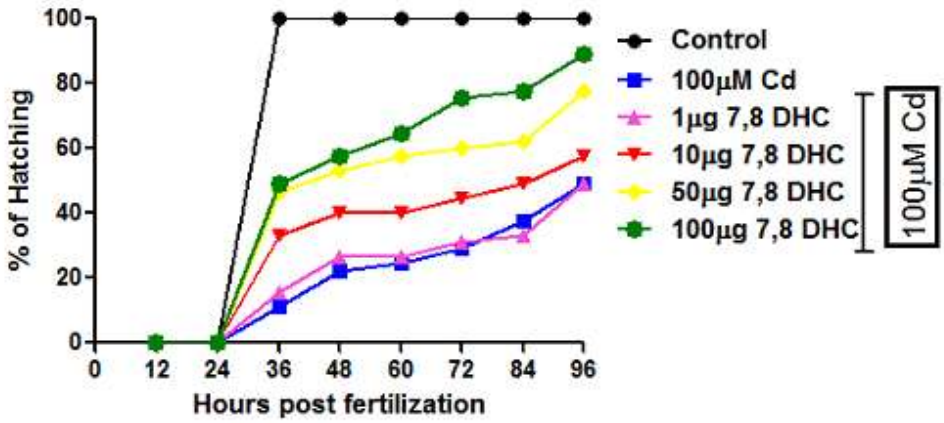

Fig. 4: Effect of 7,8 –DHC on hatching success of zebrafish embryos after exposed to Cd toxicant. Experiments were performed in triplicate and the values are represented as mean ±SEM

Developmental toxicity screening: Microscopic clarifications revealed embryos treated with Cd at the dose of 100 µM exhibited severe alterations in the developmental process. The control embryos exposed to embryo medium hatched normally without any sign of aberration. Embryo intoxicated with Cd (100 µM) exhibits significant lethal effects including yolk sac edema (YSE), a defect in the eyes (E), head (H), bent spine (BS), pericardial edema (PE), tail (T) curvature and tail tip (TT).

The co-treatment with 7,8-DHC (1, 10, 50 and 100 µg) showed significantly decreased phenotypic malformations in embryos at 24, 48, 72 and 96 hpf whereas embryos exposed to 50 and 100 µg of 7,8 –DHC showed (Fig. 6) complete salvage of Cd induced phenotypic alterations. This result represents the protective effect of 7,8-DHC against Cd toxicity and this effect might be due the antioxidant-mediated stabilization of free radicals, thereby minimizing the damage caused by free radicals.

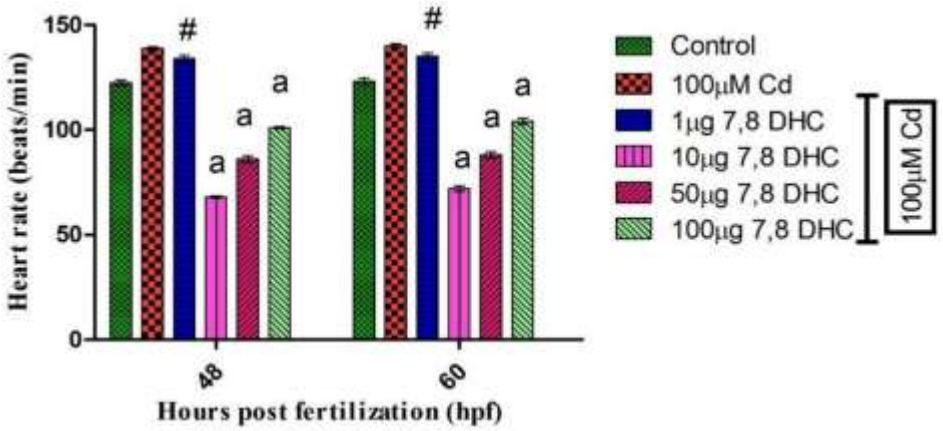

Fig. 5: Heart beat rate in zebrafish embryo exposed with Cd and Co-treated with 7,8-DHC (1, 10, 50 and 100 µg/ml) at 48 and 60 hrs time intervals. Values represent the mean ± SEM for three replicates (one way ANOVA followed by Dennett's test). Values that are significantly different at P<0.05.Comparisons:[a] P<0.05 vs Cd,[#] non-significant vs Cd treated embryo.

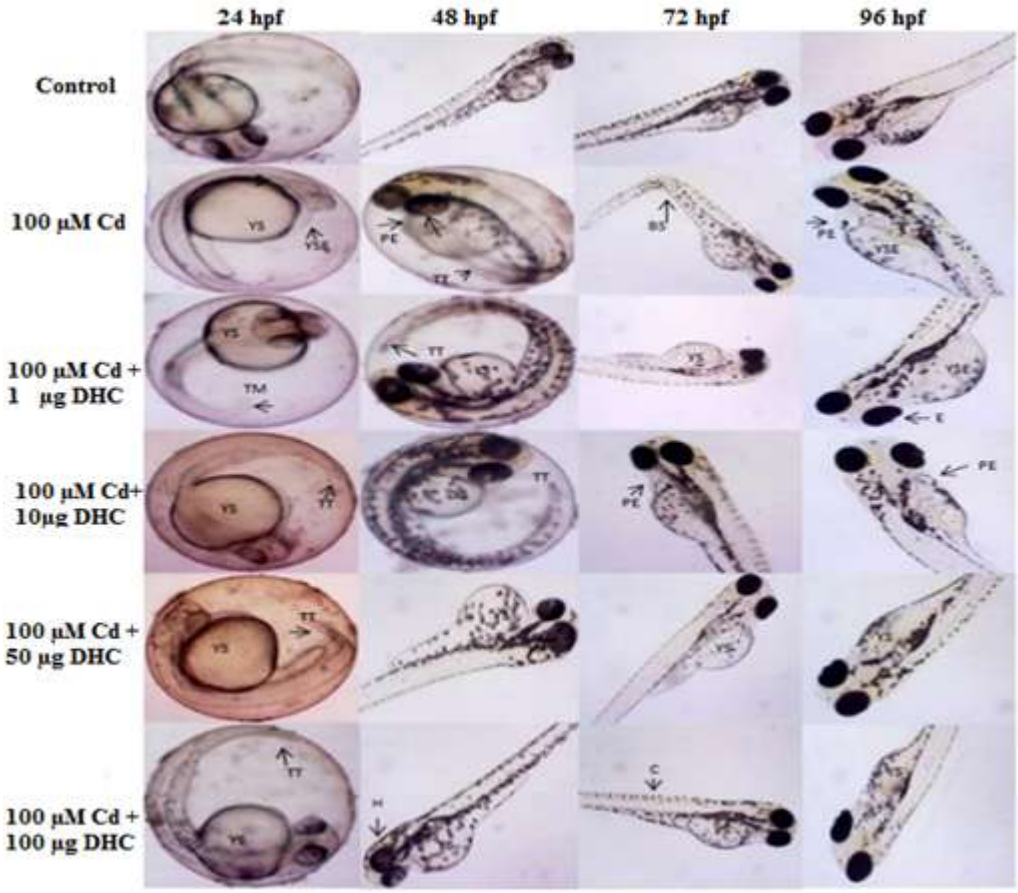

Fig. 6: Microscopic analysis of zebrafish embryos pre-exposed to Cd intoxicant and co-treated with varying concentrations of 7, 8 –DHC for 96 hpf (Magnification x4).

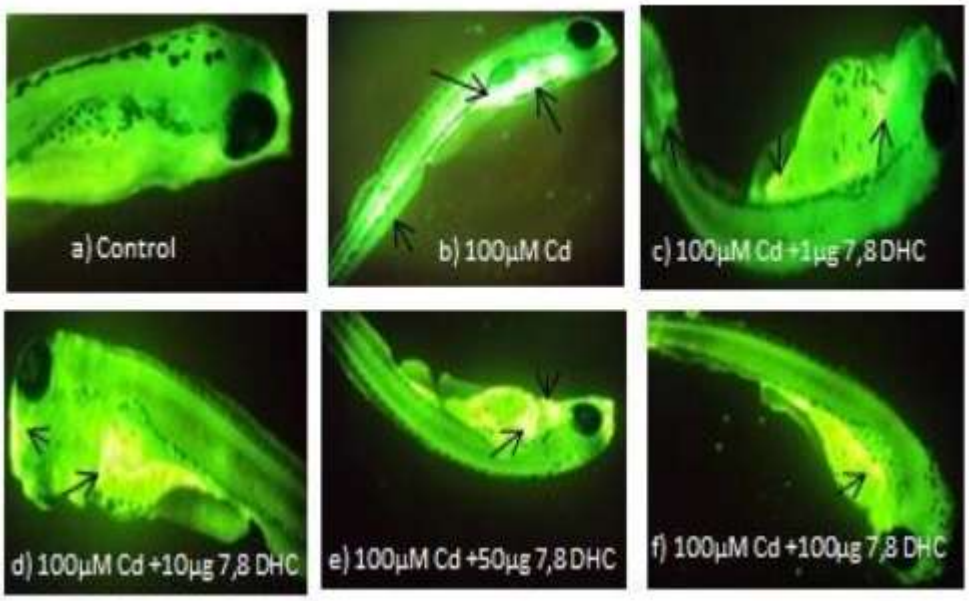

Fig. 7: Fluorscent microscopic image represents the anti-apoptotic effect of 7, 8-DHC against Cd intoxicated zebrafish larvae at 96 hpf (Magnification x4).

Apoptotic inhibitory effect of 7,8-DHC on Cd intoxicated embryos: Oxidative stress-induced cell death remains a major concern in heavy metal induced toxicity. Here, the control group generated a clear image whereas the Cd (100µM) alone treated group showed a marked increase in the fluorescence signal, suggesting that teratogenic effect incurred during Cd exposure in the zebrafish. However, in zebrafish treated with 7,8 –DHC and co-treated with 100µM Cd exposure, a dramatic reduction was observed in the amount of apoptotic cells. Besides, we determined 100 µM Cd induced cell death by measuring acridine orange fluorescence intensity in the body of the zebrafish. The increased number of apoptotic cells determining the toxic effect of Cd was gradually decreased in 50 and 100 µg 7,8-DCH. The results showed that particularly 100 µg of 7,8-DHC showed fluorescent intensity profile similar to control, representing the anti-apoptotic property of 7,8 –DHC (Fig. 7).

Histopathological Studies: The histological alterations in the notochord segments of all experimental embryos were illustrated in fig. 8. The notochord plays an important structural role that supports locomotion in larval fish. Moreover, during the embryonic development, the notochord is the site of signal origin involved in patterning axial structures such as the somites and overlying neural tube. The sections of control embryo showed regular pattern of notochord segments. Exposure with Cd intoxicant showed severe poorly organized or aligned notochord segments including the zone of horizontal myoseptum (hm) and somatic muscle (sm).

However, 7,8-DHC treated zebrafish larvae showed dose-dependent recovery of dysreregulated notochord regions when compared to Cd intoxicated larvae. This result often demonstrated the potential rescuing effect of 7,8-DHC against heavy metal toxicity.

Discussion

The acute overload of Cd is implicated to generate reactive free radicals such as superoxide anion (O_2•−), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and lipid peroxides (•L) etc. and thus causing severe detrimental effect on the aquatic and mammalian life forms. Cd is known to be a weak redox active metal, therefore, the generation of ROS was further mediated by other indirect mechanism like glutathione depletion, Kupffer cell activation, inflammation and involvement of iron for the Fenton reaction[30].

The results from our study such as DPPH, FRAP,and H_2O_2 assays suggested that 7,8-DHC could effectively neutralize the radical ions in a dose dependent manner as compared to the known standard Quercetin. This effect might be due to its ability to donate hydrogen to a free radical in order to remove odd electron which is responsible for the radical's reactivity[19]. Also the IC50 values of 7,8-DHC and standard Quercetin showed a huge gap, clearly indicating that the formulation is potent in scavenging free radicals *in vitro*. This activity is believed to be mainly due to their redox properties[1,5,39].

Few studies reported that the long-term exposure of Cd at minimum dosages is not necessarily associated with Cd-induced chronic toxicity and carcinogenesis. However, acute exposure for long term causes chronic cellular damage such as oxidative DNA damage, subsequent apoptotic resistance, epigenetic DNA methylation status changes and aberrant gene expressions etc. In our study, we mimicked the chronic conditions via exposing Cd at higher lethal concentration of 100 microgram level. By pre-treating the embryos with

higher dosage concentration of Cd, we observed the chronic developmental deformities in the developing embryos and thus, validating its lethal effects in the model system.

In survival analysis, we observed that Cd intoxicated embryos exhibited drastic decline in survival rate of zebrafish embryos. We believe that the reduction in survival rate might be due to the strong oxidative capacity of Cd[15] whereas, treatment with 7,8-DHC significantly enhanced the life span of zebrafish embryos at higher dosage exposure and improved the survival rate depicting its protective effect against Cd toxicity and it might be its strong redox scavenging ability. Thus our study corroborated with the few reports demonstrating the antioxidant potential of coumarin family in the elimination of radical ions[29].

Earlier reports showed that entrapment of metals in the chorion of the embryo induces physical stress resulting in reduced movement of embryos leading to hatching delay[2].We also found in our study that pre-treatment with Cd caused delayed hatching ability in embryos. On the other hand, 7,8-DHC treated groups showed increasing hatching ability. Thus might be due to the synergistic effect due to the endogenous antioxidant enzymes and thiol-based antioxidants participation integrally in the overall redox defense during redox challenged cellular environment.

The formation of the heart in the zebrafish provides a useful platform for the investigation of teratogenic and toxic effects. Indeed, the heart rate has been used as marker in assessing cardiac function of fish[11].Our results depicted that embryo intoxicated with Cd showed increased heart rate at 48 hpf and 60 hpf when compared to control, whereas the antioxidant 7,8-DHC treated groups showed decreased heart beats. We also found that the concentration at 100µg 7,8-DHC treated group showed normalized heartbeat compared with other lower concentrations of DHC.It is likely that the Cd interacted with reactive thiols in the mitochondrial membrane and generated ROS by modulating the electron transport chain, increasing the heart rate[10].

Our results correlated with reports showing the flavonoid group of taxifolin effectively restored Cd driven heart rate deregulation in zebrafish larvae with its antioxidant and metal chelating properties[23].Therefore, our study also strongly substantiated the metal chelating property by the 7,8-DHC which further drives rescuing potential of Cd in the intoxicated embryos.

Our apoptotic assay (AO stain) revealed that the malformation occurred mainly in the heart zone of Cd intoxicated larvae suggesting that the developing heart may be a potential target for Cd toxicity in zebrafish. Besides, there was a significant reduction in the heart rate after Cd exposure found to be correlated with the relatively increased rate of apoptotic cells in heart region. Additionally, the poor underdeveloped heart hindered the other cardiac functions leading to abnormal heartbeat, circulation failure and subsequently resulting in growth retardation due to insufficient nutrients[35].These signatures of apoptotic events were significantly decreased in antioxidant 7,8-DHC treated groups. It was confirmed that coumarins with OH groups in positions 7 and 8 were potent scavengers of O_2^- radicals and thereby render the potential of reducing the cell damage in Cd intoxicated zebrafish larvae.

Conclusion

Our overall findings demonstrated the developmental toxicity in zebrafish embryos after exposure to Cd. The observed developmental toxicities were the resultant of general stress responses of embryos exposed to toxicants. Interestingly, we found in our study that the toxicities induced by Cd metal were significantly rescued upon treatment with 7,8-DHC. This property can be further extrapolated in the environmental removal of Cd from the effluents and thus preventing the teratogenic effects of Cd toxicity and preserving the biodiversity.

Acknowledgement

We gratefully acknowledge our colleagues in the zebrafish field for their collective contributions, with special thanks to all members of Dr. Elangovan Lab. We appreciate Dr. Krishnan Manigandan, Lab No. 2304, Lab of Genetic Engineering, Department of Biotechnology, Daegu University, Gyeongbuk 38453, Republic of Korea for critical reading of the manuscript. This research did not receive any specific grant from funding agencies in the public or commercial sectors.

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(Received 26th May 2021, accepted 04th August 2021)