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Induction of DNA Damage and GADD45β gene Mutation in Zebra fish (*Danio rerio*) due to Environmentally Relevant Concentrations of Organochlorine Pesticides & Heavy Metals

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ABSTRACT: Zebra fish were exposed to environmentally relevant concentration of pesticides and metals for a period of 14 days. The individual and the combined toxicity of pesticides and metals were studied. Damage caused to the DNA and induction of mutation in the GADD45β gene was investigated in this study. The present investigation revealed that exposure of zebra fish to pesticides and metals induced DNA damage and also mutation in the GADD45β gene. The pesticides treated group (III) showed significantly higher damage followed by metals treated group (II). The DNA damage was comparatively less in the pesticides and metals exposed group (IV). GADD45β gene which is considered to be one of the most important gene involved in G phase arrest, has been found to be mutated in zebra fish when exposed to metals and pesticides. The comparison of amplified gene sequence of GADD45β gene with the sequence of the zebra fish retrieved from the NCBI confirmed the mutation in the gene sequence of zebra fish treated with pesticides and metals. The induction of DNA damage in fish due to contaminants may lead to mutations, which contribute towards the multistage carcinogenesis process.

Key words: Zebra fish, Pesticides, Metals, DNA damage, Gene mutation

INTRODUCTION

Fish are particularly sensitive to the influence of pesticides and other toxic pollutants because they are able to uptake and retain the dissolved xenobiotic in water via active or passive processes. The use of physiological and biochemical parameters as indicators of water quality has recently been developed to detect sublethal impacts of pollutants.A number of intrinsic and extrinsic mutagens cause structural changes in cellular DNA that could eventually lead to alteration in the coding properties of DNA and cellular toxicity. Free radicals produced by oxidative processes can attack DNA at bases or sugars, causing primarily single strand breaks, as well as, secondary double strand breaks (Ferri *et al.,* 1994; Sarker *et al.,* 1995; Spencer *et al.,* 1996). DNA strand breakage is not an uncommon occurrence in a cell. Heat energy causes a number of abasic sites per cell per day which, however, are rapidly repaired. This is an example of insult to DNA that indirectly results in the strand breakage (i.e., initial damage is the loss of a base from the DNA chain, the repair of this damage results in a temporary gap in the DNA molecule). DNA strand breaks are potentially pre-mutagenic lesions (Kammann *et al.,* 2001) and may provide a meaningful indication of the degree of the oxidative damage of the DNA (Imlay *et al.,* 1988). Biomarkers of DNA damage are valuable tools to assess acute and chronic exposure of aquatic organisms to genotoxic substances. Moreover, as genotoxins may induce changes in DNA that are passed on to future generations, this kind of biomarker can be used in a predictive way, avoiding irreversible ecological consequences (Monserrat *et al.,* 2006). They have been induced in the fish by *in vitro* and *in vivo* exposure to chemicals such as benzo (a) pyrene, metals and PCBs (Everaarts, 1995; Mitchelmore and Chipman, 1998). In fish, DNA repair mechanisms are not well studied, when compared to that of mammals. Any defect in DNA repair mechanisms leads to genomic instability, either as mutations or other genetic alterations. Small fish model, zebrafish has been used in cancer studies for many years (Amatruda *et al*., 2002). A great deal of zebrafish genomics information is available online through dedicated portals. Similarly, studies using medaka in chemically-induced carcinogenesis have shown promising results (Reddy *et al*., 1999; Liu *et al*., 2003). As most occupational and environmental exposures to pesticides are to

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mixtures, the genotoxic potential evaluated on single compounds could not be extrapolated to humans. So, the present investigation aims at studying DNA damage and mutation in the GADD45β gene due to individual and combined toxicity of pesticides and heavy metals in Zebra fish so that the result can be extrapolated to human beings.

MATERIALS & METHODS

Zebrafish (*D. rerio*) were purchased from a commercial fish supplier and acclimatized for one week in the laboratory conditions in 50 L glass tanks before the start of the experiments. Four different groups (each 20 animals of mixed sex) were used for the experiment: control with acetone (Group I), Group II represents the mixture of metals such as Ni in the form of Nickel chloride (0.05 mg/L), Cd in the form of Cadmium chloride (0.02 mg/L) and Pb as Lead nitrate (0.32 mg/L). Group III comprises the mixture of six pesticides Viz., α-HCH (47 ng/L), o,p'-DDE (46.4 ng/L), aldrin (36 ng/L), dieldrin (16 ng/L), heptachlor (259 ng/L) and mirex (17.5 µg/L). Group IV includes mixture of both metals and pesticides. The concentration of pesticides and metals used for the toxicity study reflect those detected in the surface water of River Cauvery and Veeranam Lake (Bhuvaneshwari, 2011). The stock solutions of pesticides were prepared using acetone and the metals were prepared in deionized water.

DNA strand breaks were quantified according to De Lafontaine *et al.,* (2000). Tissue homogenates (50 µl) were incubated in 500 µl of a SDS solution (2%) containing 50 mM NaOH, 10 mM Tris and 10 mM EDTA and 500 µl of 0.12 M of KCl at 60°C for 10 min. Samples were cooled to 4°C for 15 min in order to precipitate SDS associated nucleoproteins and genomic DNA. The mixture was further centrifuged at 8000g for 4 min to enhance the precipitation process. Levels of single and double stranded DNA remaining in the supernatant were labeled by mixing 50 µl of supernatant with 200 µl of 1 µg/ml. Heochst dye (bis Benzimidett 33258, Sigma-aldrich, India) in 0.1 M phosphate buffer (pH 7.4). The resulting complex was then measured using an excitation/emission wavelength of 360/450 nm. Genomic DNA was extracted by grinding the whole body tissue of zebra fish in a mortar and pestle with 1 ml of extraction buffer (10 mM Tris-Cl pH = 8, 1% SDS, 0.4 M NaCl, 5 mM EDTA). Then 50µl proteinase K solution (0.01 g/ml) was added and the mixture was incubated for 30 min at 37 ºC and subsequently for 60 min at 65ºC. The solution was twice extracted with equal volumes of phenol-chloroform-isoamylalcohol (25:24:1) and finally with chloroform-isoamylalcohol (24:1). DNA was precipitated from the final aqueous phase by adding 750 µl of 96 % ethanol and 250 µl of 3M ammonium acetate and placing the mixture at - 70ºC for 30 min. The precipitated DNA was pelleted in a micro centrifuge and dried under vacuum at room temperature. The pellet was resuspended in 50 µl of TE-buffer (0.01 M Tris-Cl pH = 7.5, 0.001 M EDTA) and stored at 4ºC. The nucleic acid concentration was measured in a spectrophotometer at 260 nm. A fragment of the Gadd45beta gene was amplified by PCR. The PCR primers used were 5'-TCACAGTCGGCGTTTATGAG-3' (Forward Primer) and 5'-GATGTCGTTATCGCAGCAGA-3' (Reverse Primer). Amplified in PCR with optimized conditions and amplicons were run on a 1% agarose gel for confirmation, containing 1 µg/ml ethidium-bromide, at room temperature at 50 V in TBE running buffer. A 100 bp DNA marker was used as reference. The gel was photographed under UV-light. The amplified PCR fragments were purified and after purification, direct sequencing was carried out by the dideoxy chain termination method using a Perkin Elmer ABI 3100 sequence analyzer (Applied Biosystems, Foster City, CA, USA). Antisense primer was used as the sequencing primer.

RESTULTS & DISCUSSION

The genotoxic effect of pesticides and metals in the zebra fish was assessed through the alkaline precipitation assay in the present study.Fig.1 represents the DNA damage in the control and the treated groups. The extent of DNA damage was high in group III (pesticides treated) followed by metals exposed group (II). Since the DNA damage can also occur due to various normal metabolic processes inside the cell at a rate of 1,000 to 1,000,000 molecular lesions per cell per day (Lodish *et al.,* 2004), the control group also exhibited the DNA damage. The pesticides and metals treated group (IV) showed DNA damage similar to the control. The results of the present study coincide with De Lafonatine *et al.,* (2000) who observed significant DNA damage in zebra mussel (*Dreissena polymorpha*) from St Lawrence river (Canada) contaminated with metals. The detected DNA damage could be due to cytotoxic and/or genotoxic effects. Exposure to genotoxic compounds could induce DNA damage not only directly but also through other mechanisms, such as oxidative stress or inflammatory processes (Lebailly *et al.*, 1998). Bolognessi *et al.,* (1999) studied the heavy metal induced oxidative stress in the mussels and observed that Cd induced statistically significant increase of DNA damage. Zeljezic and Vrhovac, (2001) studied that the mixture of pesticides such as atrazine, alachlor, cyanazine, 2,4-dichlorophenoxyacetic acid and malathion caused increase in the level of DNA damage. DNA strand

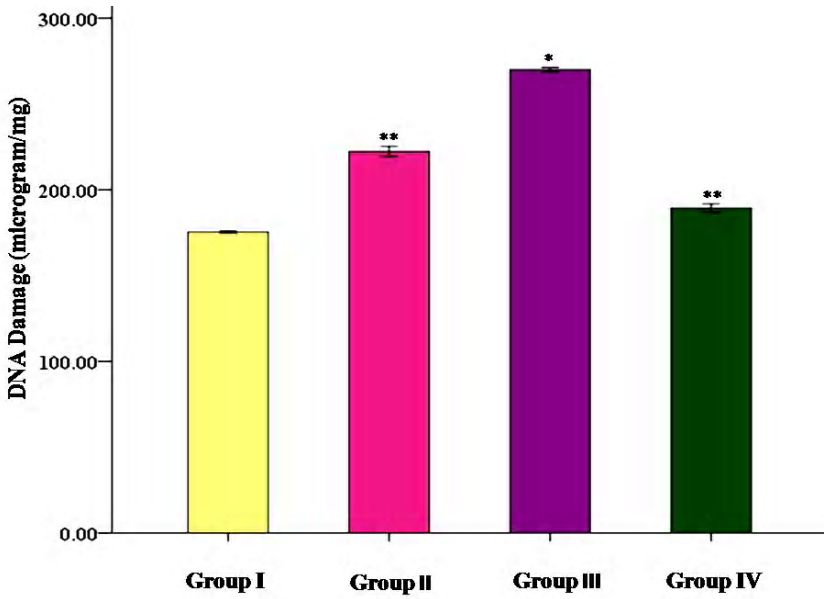

Fig.1. DNA Damage (microgram/mg) in zebra fish exposed to metals and pesticides. Data are expressed as mean ± standard deviation. * : Very highly significant at p<0.001; **: highly significant at p<0.01

breaks and apoptosis in erythrocytes of the European eel *Anguilla anguilla* were observed from in vivo exposure to B[a]P, Arochlor 1254, and dioxin (Regoli *et al.,* 2003). These carcinogenic compounds are known to act through oxygen free radical mechanisms (Nigro *et al.,* 2002). One of the cellular targets of ROS is DNA. More than 100 different types of oxidative DNA lesions have been described, ranging from base modification to single- and double-strand DNA breaks and interstrand cross-links. These lesions disrupt vital processes such as transcription and replication, which may cause cell death or growth arrest or may induce mutations that lead to cancer (Hasty *et al.,* 2003).

The GADD45 gene can be induced by ionizing radiation and several DNA-damaging xenobiotics (Blaszyk *et al.,* 1996). The gadd genes were also found to be inducible by various types of DNA damage. GADD45ß has been associated with control of cellular growth and apoptotic cell death. The *GADD45* gene is induced when cells are subjected to DNA damage leading to arrest in the G~ phase of the cell cycle (Kastan *et al.,* 1991). The results of the present investigation showed that pesticides and metals have induced mutation in the GADD45β gene. Genbank DNA sequence data (Accession no. emb|CU062501.10|) was used for comparison obtained in this study. The GADD45β sequences obtained in the present study displayed all the conserved structural domains of the gene and showed a high degree of similarity with the genes sequence available with the Genbank (Fig. 2a). The exposure of zebra fish to metals (group II) showed 97% similarity with deletions of four adenine (A) bases and transversion mutation i.e conversion of T to A was observed (Fig. 2b). The pesticide exposure (group III) (Fig. 2c) also resulted in deletions of four bases that includes adenine, guanine and thymine bases and exhibited transition mutation (G to A) whereas in the pesticides and metals treated group (IV) (Fig. 2d) insertion of cytosine base and transition mutation (G to A) was observed. The mutations occurring at hot spots may produce altered proteins that lead to deregulated activation of the GADD45β protein. Due to the deregulated activity of GADD45β protein, the G phase process continues without arrest during neoplasia in stressful conditions thus resulting in tumour. Both transitions and transversions can change the amino acid composition of the corresponding protein, but the biochemical difference in the protein product tends to be greater for transversions. These mutations in the cDNA of GADD45β fall within a short sequence that appears to be conserved and also falls under the exon region that contains the open reading frame (ORF). The ORF is the region that potentially encodes a protein. Deletions and insertions can change the reading frame of the mRNA downstream of the mutation, resulting in a frameshift mutation. When the reading frame is shifted, incorrect amino acids are usually incorporated. Frameshifts may bring stop codons into the reading frame, creating a shortened protein and it may also result in read-through of stop codons, resulting in a longer protein. Frameshift mutations result from insertions or deletions when the

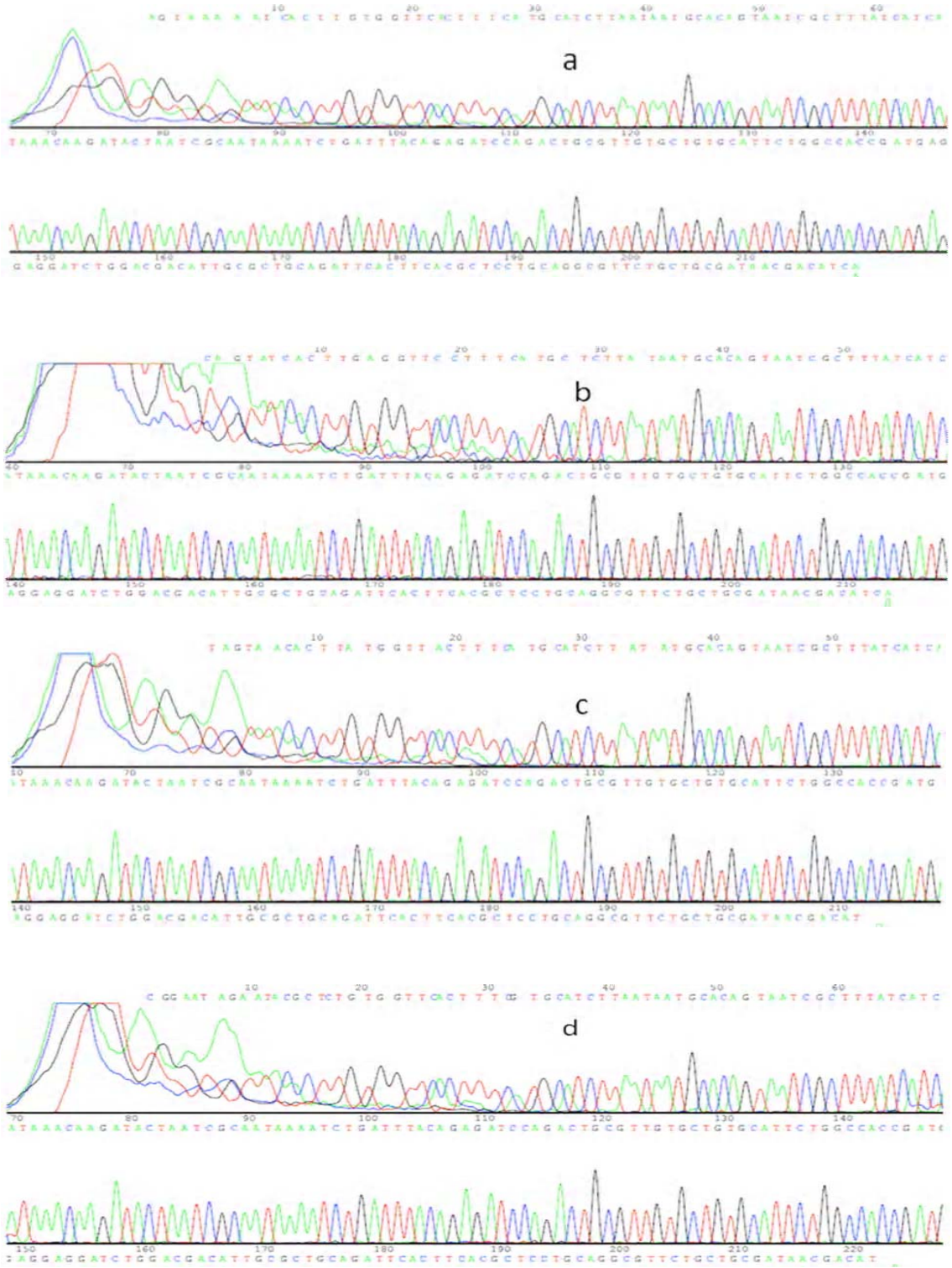

Fig. 2. Chromatogram of GADD45β gene sequence a. Group I (Control) b. Group II (Metals treated) c. Group III (Pesticides treated) d. Group IV (Pesticides & Metals treated)

number of affected base pairs is not divisible by three. Oxidants such as free radicals or hydrogen peroxide produce multiple forms of damage, including base modifications and can produce point mutations, insertions and deletions from the DNA sequence, as well as chromosomal translocations. Apoptosis occurs in certain cell types after treatment with genotoxic agents. Since these treatments also induce the GADD genes, it has been suggested that the GADD genes may be involved in apoptosis as well as in growth arrest after DNA damage (Hollander *et al.,* 1997). Correlation existed between increasing concentrations of heavy-metal ions in the media and increase in both uptake into the plant (*A. thaliana*) and levels of point mutations. The frequency of homologous recombination and point mutations reflected the level of DNA damage produced by heavy metals (Olga *et al.,* 2001). Oncogenes, such as *ras*, activated by point mutations, have been identified as genetic targets both in feral fish from heavy contaminated areas as in laboratory exposed fish (Rotchell *et al.*, 2001). Mutations in other genes such as smad4, p16 and Gadd45 have also been identified in pancreatic carcinogenesis (Yamasawa *et al.,* 2002). Hoyer *et al.,* (2002) showed higher risk of breast cancer with mutated p53 among women exposed to high levels of dieldrin and PCBs. Howsam *et al.,* (2004) assessed point mutations in K-ras and p53 genes in tissue samples by polymerase chain reaction/single-strand conformation polymorphism. Mono-ortho PCBs were further associated with transversion-type mutations in both genes. Howsam *et al.,* (2004) also observed that compounds such as *p,p´*-DDE and α-HCH that had an overall moderate association with colorectal cancer showed a significant increase in risk for tumors with mutation of the p53 gene. The mutations in the K-ras proto-oncogene and p53 gene are the most common DNA sequence alterations (Maitra *et al.,* 2006) in the stress conditions. Nickle, a toxic, mutagenic and carcinogenic metal resulted in G-to-T transversions in codon 12 of the K-ras gene in rat renal sarcoma induced with nickel subsulfide (Higinbotham *et al.,* 1992). The differences in K-ras and p53 mutations may be associated with high levels of environmental exposures, such as organochlorine pesticides or heavy metals, especially in the heavily polluted region (Kriegel *et al.,* 2006).

CONCLUSION

The present study showed that there is direct correlation between the DNA damage and exposure of zebra fish to metals and pesticides. The GADD45β gene which is considered to be one of the most important genes involved in G phase arrest has been found to be mutated in zebra fish when exposed to metals and pesticides. The mutations occurring at hot spots may produce altered proteins that lead to deregulated activation of the GADD45β protein. Due to the deregulated activity of GADD45β protein, the G phase process continues without arrest during neoplasia in stressful conditions thus resulting in tumour.

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