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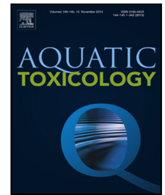

Gene expression in *Catla catla* (Hamilton) subjected to acute and protracted doses of gamma radiation

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ABSTRACT

Studies on transcriptional modulation after gamma radiation exposure in fish are limited. Cell cycle perturbations and expression of apoptotic genes were investigated in the fish, *Catla catla* after acute and protracted exposures to gamma radiation over a 90 day period. Significant changes in gene expression were observed between day 1 and 90 post-exposure. Gamma radiation induced a significant down-regulation of target genes gadd45α, *cdk1* and *bcl-2* from day 1 to day 3 after protracted exposure, whereas it persists till day 6 upon acute exposure. From day 12 onwards, *Gadd45α*, *cdk1* and *bcl-2* genes were up-regulated following protracted exposure, indicating DNA repair, cell-cycle arrest and apoptosis. There exists a linear correlation between these genes (*gadd45α* – $r = 0.85$, $p = 0.0073$; *cdk1* – $r = 0.86$, $p = 0.0053$; *bcl-2* – $r = 0.89$, $p = 0.0026$) at protracted exposures. This is the first report on the dual role of *bcl-2* gene in fish exposed to acute and protracted radiation and correlation among the aforementioned genes that work in concert to promote 'repair' and 'death' circuitries in fish blood cells.

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1. Introduction

The ever increasing demand of nuclear energy for power generation coupled with the problem of intentional or accidental release of radioactive waste pose serious concerns on the protection of ecosystems. Moreover, the environmental risk assessment of chronic exposure to ionizing radiation is currently hampered by the lack of reliable experimental knowledge and hence based on extrapolation from data obtained for acute exposures (Copplestone et al., 2001). Radiation-induced health effects on aquatic organisms are in fact a consequence of chronic exposures except accidental outbreaks. A few studies have addressed the effects of ionizing radiation on the transcriptional activity in fish and on time course expression of stress response genes (Song et al., 2014; Smith et al., 2007; Salbu et al., 2008; Dowd et al., 2006; Lyng et al., 2004; Rhee et al., 2012) and fish embryos (Jaafar et al., 2013). However, studies on expression profile of genes involved in fundamental physiological processes like cell cycle after gamma radiation in fish are lacking.

DNA damage and repair activities provoked by gamma radiation on aquatic biota are important because unrepaired DNA lesions have the potential to be mutagenic, cytotoxic and carcinogenic. Since the primary target of ionizing radiation is the DNA molecule, double strand breaks (DSBs) and reactive oxygen species (ROS) could activate DNA repair systems to maintain the genomic integrity or induce apoptotic pathways to eliminate severely damaged cells. Cellular response to DSBs elicits a powerful signalling network that coordinate a number of cellular systems in a hierarchical process executed in a controlled manner (Ciccia and Elledge, 2010).

Gadd45α is a member of a group of genes whose transcript levels are enhanced following stressful growth arrest conditions and exposure to genotoxicants. Transcription of this gene is mediated by both *p53*-dependent and independent mechanisms (Libermann and Hoffman, 2008). Cyclin dependent kinase 1 (*cdk1*) also known as cell division control protein 2 (Cdc2) is a highly conserved protein with a pivotal role in cell cycle regulation, cellular differentiation, morphogenesis, versatility and adaptability of any organism under xenobiotic exposures (Enserink and Kolodner, 2010). B-Cell lymphoma (*bcl-2*) belongs to the family of apoptosis regulatory proteins encoded by the *bcl-2* gene. The Bcl-2 protein family includes several anti- and pro-apoptotic proteins assumed to be of special

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importance during ionizing radiation responses (Belka and Budach, 2002).

In an earlier attempt to elucidate specific biomarkers of ionizing radiation exposure in aquatic sentinels, a modified erythrocyte micronucleus cytome assay (EMNCA) was standardised in the authors' laboratory in the fish *Catla catla* (Anbumani and Mohankumar, 2012). Alike in humans, several cytogenetic biomarkers such as nucleoplasmic bridges, tailed nuclei and fragmented nuclei were observed revealing DNA damage and apoptotic signatures after exposure to ionizing radiation. A specific cytogenetic signature of ionizing radiation, nucleoplasmic bridges noted was proposed as a probable biomarker. These findings incited us to study the effects at the molecular level by observing the expression of genes involved in DNA damage and cell cycle.

There is lack of scientific knowledge on the impact of ionizing radiation on the expression profile of genes with reference to aquatic organisms like fish, which occupy a key role in the aquatic food chain. The tissues affected upon ionizing radiation exposure are gills, liver, kidney and blood. However, blood cells are preferred for repeated sampling and long-term observations for tumour development and mortality in field studies and hence, in the present study an attempt was made to analyze the expression profile of genes involved in DNA damage and cell cycle regulations after acute and protracted exposures to gamma radiation in blood cells of the fish *Catla catla* under controlled laboratory conditions.

2. Materials and methods

2.1. Experimental fish specimens

Freshwater fish *Catla catla* was chosen as a test system since it is a commonly available edible species of considerable economic importance and proven to be a sensitive indicator of environmental stress (Tilak et al., 1981). Healthy specimens having an average wet weight and length of 10 ± 2.0 g and 11 ± 2.0 cm respectively were procured from a fish farm and subjected to a prophylactic treatment in 0.05% potassium permanganate solution for 2 min to avoid any dermal infections. They were then acclimatized for 21 days under laboratory conditions with natural photoperiod and fed with rice-bran oil cake. The fecal matter and other waste materials were siphoned off daily to reduce the ammonia content in water. Water quality parameters were maintained within the normal range (pH – 7.2 ± 0.05, DO – 8.8 ± 0.02 mg/L, Temp.:- 24 ± 1 °C and hardness-in terms of $CaCO_3$ were 190 ± 0.2 ppm respectively).

2.2. Exposure to gamma radiation

Acclimatized fish were exposed to acute and protracted doses of gamma radiation and the exposure details were described in detail elsewhere (Anbumani and Mohankumar, 2012). Briefly, fish were placed in glass beakers of 1 L capacity containing dechlorinated tap water. The beakers were placed at a radial distance of 16 cm from a sealed Cs-137 gamma source (1 Ci, BRIT, Mumbai, India) and irradiated for 42 h at a dose rate of 0.002 Gy/min to a total dose of 5 Gy. The irradiation set-up could accommodate about 10–12 beakers in a circle to provide uniform irradiation. The size of the glass beakers was kept small to ensure that all fish were given approximately the same radiation dose. Another set of fish was irradiated at a dose rate of 3.2 Gy/min to a total dose of 5 Gy in a gamma chamber containing a sealed Co-60 gamma ray source (GC-900, BRIT, Mumbai, India). Fish from the same lot were sham exposed and used as controls. After irradiation, fish were transferred to glass aquaria of 50 L capacity containing dechlorinated tap water. Fish exposed to a dose rate of 3.2 Gy/min is considered acute and 0.002 Gy/min protracted.

Peripheral-blood was collected from anesthetized fish (MS222 as anesthetic agent at a concentration of 500 mg/L) at different duration intervals (days 1, 3, 6, 12, 18, 30, 45, and 90) at the rate of five fish per interval, by severing the caudal peduncle using EDTA as an anticoagulant (2.7%). Since the specimens used in the present study were fingerlings, repeated blood sampling was difficult and for the sake of practical convenience specimens were randomly selected, sampled and discarded. Exposure related mortality was not observed throughout the study period.

2.3. Isolation of RNA and reverse transcription

RNA from the whole blood was extracted using Ribopure RNA kit (Invitrogen) following the manufacturer's recommendations. The RNA concentration was estimated based on absorbance at 260 nm using Biophotometer plus (Eppendorf, Germany). RNA quality was verified by electrophoresis using agarose gels (1.5%) stained with ethidium bromide, and ensuring 260/280-nm OD ratios between 1.8 and 2.1.

Reverse transcription was performed with pooled mRNA samples ($n = 5$) belonging to each time interval for both sham-irradiated and irradiated groups using Tetro cDNA synthesis kit (BIOLINE, GmbH, Germany) following the manufacturer's instructions. Total DNA-free RNA (1 μg) was heat-denaturalized and reverse transcribed at 37 °C for 60 min in 20 μL reaction buffer (50 mM Tris-HCl, pH8.6, 40 mM KCl, 1 mM $MnSO_4$, 200 μm random hexamers and 200units of reverse transcriptase) as template. The reaction mix was incubated at 65 °C for 10 min to achieve enzyme inactivation and then placed on ice and subjected to PCR (polymerase chain reaction) with 5 x RT buffer, RNase inhibitor and reverse transcriptase enzyme following the manufacturer's instructions.

2.4. Quantitative real time-PCR (QRT-PCR)

Genes exhibiting differential expression were confirmed by quantitative real time-PCR. Primer sequences (Table 1) were designed based on the Zebrafish mRNA sequences available in the DDBJ/Gen Bank TM/EBI data bank using the Primer 3 software (http://frodo.wi.mit.edu/primer3) obtained from GenScript, United States. PCR amplifications were performed in a total reaction volume of 35 μL with 10 μM of each primer, 17.5 μL of 2× SYBR Green PCR Master Mix (BIOLINE, GmbH, Germany), 3.5 μL of cDNA template and 6.48 μL of sterile water. All qRT-PCR assays were run in triplicates with Realplex Eppendorf (epgradient) Master cycler (Germany) using SYBR green. Reaction conditions were: 10 min at 95 °C, followed by 40 cycles of PCR amplifications (denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s and extension at 72 °C for 15 s). qRT-PCR data were analyzed using the Eppendorf Master Cycler Realplex software. The threshold cycle (Ct) value represents the cycle number at which the sample fluorescence level rises above

Table 1
Primer sequences, Genbank accession numbers used for quantitative real-time rtPCR.

Gene	Function	Genbank accession	Forward primer (5′–3′)	Reverse primer (5′–3′)
18s r RNA	18S Ribosomal RNA	Generic	CGGTGAACCTTGGTGACTCT	CTTGGATGTGGTAGCCGTTT
Gadd45α	Cell cycle arrest & DNA damage	NM001002216	TTGAAGAACCGTGTGGAGATAAC	TGTTCACTCGCAAGATATTGATG
cdk1	Cell cycle arrest	CU062631	GATCCTACGTTCACTCGGTAATG	TTGGCTTGGTAGAAATCTGTGAT
bcl-2	Apoptosis	BC133848	TTGTGGAGAAATACCTCACGCAT	GAGTCTCTCTGCTGACCGTACAT

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