MOLECULAR CYTOGENETIC STUDIES ON THE TOXIC EFFECTS OF GAMMA RADIATION IN ZEBRAFISH AND COMMON CARP

Thesis submitted to Goa University
for the award of the Degree of

DOCTOR OF PHILOSOPHY
In
ZOOLOGY

By

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July 2017

Dedicated to my family and friends

CERTIFICATE

This is to certify that the thesis entitled "*Molecular cytogenetic studies on the toxic effects of gamma radiation in zebrafish and common carp*" submitted by *Mr. Praveen Kumar M.K*., for the award of the degree of Doctor of Philosophy in Zoology, Goa University, is based on the original studies carried out by him under my supervision. This thesis or any part thereof has not been previously submitted for any other degree/diploma in any other universities or institutions in India or abroad.

Date:

Prof. S. K. Shyama

(Research Guide)

DECLARATION

I, ***Praveen Kumar M.K***. hereby declare that the present thesis entitled "***Molecular cytogenetic studies on the toxic effects of gamma radiation in zebrafish and common carp***" is my original contribution and the same work has not been submitted to any other universities or tertiary institutions in India and abroad for any degree/diploma. The literature related to the problem investigated has been cited. The work was undertaken at the Faculty of Life Sciences and Environmental Sciences, Department of Zoology, Goa University. The opinions and conclusions drawn are not necessarily those of the Goa University.

Date: **Praveen Kumar M.K.**

                                                                     (Research Scholar)

ACKNOWLEDGEMENTS

I would also like to thank Mrs. Geethalakshmi K, for her support and encouragement during my Ph.D. tenure.

I would be failing in my duty if I do not express my sense of gratitude to all my well-wishers, who have either directly or indirectly helped me in various capacities in the successful completion of research thesis.

Last but not least, I would like to offer my most heartfelt thanks to my mother who kept me going with her warm and comforting words, my father and my brothers Pradeep, my sisters Premalatha and Prathibha with their kind heart and everlasting faith in me. My gratitude and thanks also go to Prasanth, Vidya, Madav, Yogesh, Sanjeeva, Ravi and Boodamma. I thank them for being there for me throughout my life and supporting my every decision in life. I love them and thank them for being the best family one could ever wish for.

Praveen Kumar M.K.

July 2017

ABBREVIATIONS

α	Alfa
β	Beta
γ	Gamma
%	Percentage
μl	Micro litre
μg	Microgram
ml	Millilitre
>	Greater than
<	Less than
^{60}Co	Cobalt 60
ANOVA	Analysis of Co-variance
DSBs	Double Strand Beaks
DDW	Double distilled water
DNA	Deoxyribonucleic acid
DMSO	Dimethyl Sulfoxide
IAEA	International Atomic Energy Agency
IR	Ionizing Radiation
ISO	International Organization for Standardization
kV	Kilo Volt
LET	Linear Energy Transfer
MN	Micronuclei
SCE	Sister Chromatid Exchange
SSBs	Single Strand Breaks
SCGE	Single Cell Gel Electrophoresis
MeV	Mega electron Volt
Gy	Gray
hpf	hours post fertilization
h	hours

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1. General introduction and review of literature

1.1. Radiation:

Radiation is a type of energy that is transmitted in the form of waves or streams of particles. Electromagnetic radiation is the type of energy that encompasses light, heat, and x-rays. All waves of electromagnetic radiation travel at the speed of light, different types of waves have vastly differing wavelengths, frequencies, and energies. The shorter the wavelength of the radiation, the greater the frequency and the larger the energy. The electromagnetic spectrum ranges from gamma (γ) radiation, which has the shortest wavelength, highest frequency and greatest energy to radio waves, which has the longest wavelength and lowest frequency and energy.
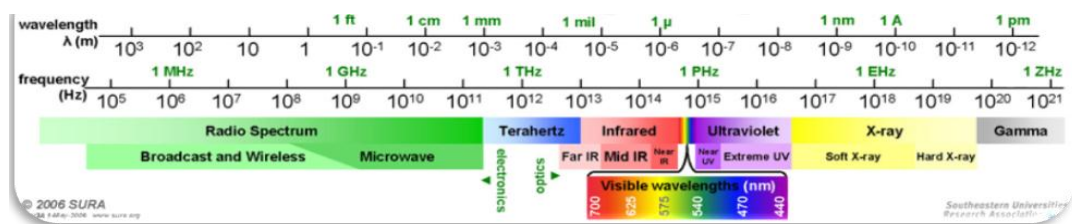
Figure 1.1: Chart of Electromagnetic radiation

Radiation is divided into two basic types according to the quantum of energy they caary and their ability for ejecting electrons from molecules:

    i. Non-ionizing radiation

    ii. Ionizing radiation

Non-ionizing radiations do not carry enough energy to ionize atoms or molecules. These waves have energies enough only to excite the atoms and molecules of the medium through which they are moving, causing them to vibrate faster. These electromagnentic waves are of longer wavelength ranging from near ultraviolet rays to radiowaves.

The ionizing radiation on the other hand, has enough energy to ionize the molecules. These radiations have high energy and short wavelength such as ultra violet radiation, x-ray and gamma rays. Alpha, beta particles and gamma rays produced by radioactive decay can cause ionization of atom and molecules of the medium through which they pass and convert them into charged ions. Ionizing radiation may be of the two types viz. directly ionizing or indirectly ionizing. Directly ionizing radiation carries an electric charge that interacts, directly with atoms in the tissue or medium exposed by electrostatic attraction or repulsion. Indirectly ionizing radiation is not electrically charged. However, the hidden energy of their radiation gets absorbed by the atom and results in charged particles.

There are many types of ionizing radiation. The following are some of the relevant ones:

i. Alpha radiation (α rays):

Alpha particles consist of two protons and two neutrons, and since they have no electrons, carry a positive charge. Due to their size and charge, alpha particles are barely able to penetrate skin and can be stopped completely by a sheet of paper.

ii. Beta radiation (β rays):

Beta radiation consists of fast moving electrons ejected from the nucleus of an atom. Beta radiation has a negative charge and is about 1/7000th the size of an alpha particle and so is more penetrating. However, it can still be stopped by a small amount of shielding, such as a sheet of plastic.

iii. Gamma radiation (γ rays):

Gamma radiation is a very penetrating type of radiation. It is usually emitted immediately after the ejection of an alpha or beta particle from the

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nucleus of an atom. Because it has no mass or charge, it can pass through the human body, but will be absorbed by denser materials such as concrete or lead.

iv. X-rays:

X-rays are a form of radiation similar to gamma radiation but they are produced mainly by artificial means rather than from radioactive substances.

v. Neutron radiation:

Neutron radiation occurs when neutrons are ejected from the nucleus by nuclear fission and other processes. The nuclear chain reaction is an example of nuclear fission, where a neutron being ejected from one fissioned atom will cause another atom to fission, ejecting more neutrons. Unlike other radiations, neutron radiation is absorbed by materials with lots of hydrogen atoms, like paraffin wax and plastics.

1.2. Units of measure and exposure:

The effects of gamma and other ionizing radiations on living tissue are more closely related to the amount of energy deposited in tissue rather than their charge. This is called *absorbed dose*. *Gray* (Gy) is defined as the amount of radiation required to deposit 1 joule of energy in 1 kilogram of any kind of matter. The SI units for absorbed dose are expressed as J/kg.

Rad is the (obsolete) corresponding traditional unit, equal to 0.01 J deposited per kg (100 rad = 1 Gy).

Sievert (Sv) is the SI unit of equivalent dose, which for gamma rays is numerically equal to the gray (Gy). The rem is the traditional unit of equivalent dose. For gamma rays it is equal to the rad or 0.01 J of energy deposited per kg (1 Sv = 100 rem).

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1.3. Radiation sources and applications:

All living organisms get exposed to at least small amount of radiation during their life span from diverse sources. The sources of gamma radiation can be divided into man-made sources and natural sources. The man-made sources of gamma radiation include nuclear weapons, bombs covered with radioactive material such as cobalt-60 (^{60}Co), nuclear power production reactors, mining of radioactive materials (krypton-85, tritium, carbon-14, and iodine- 129), accidents in nuclear reactors and nuclear waste production. Applications of nuclear technologies have made a very significant contribution to modern civilization. It is useful to humans in several ways, including as a source of power, as a medical diagnostics and also as an industrial tool.

1.4. Sources of radioactive contaminants of the aquatic environment:

Naturally occurring radionuclides, fallout from atmospheric runoff, atmospheric deposition and the radioactive effluents from medical, industrial and nuclear facilities released either accidentally or intentionally are some of the major sources of radioactivity in the aquatic environment. Nuclear accidents such as the nuclear reactor explosion in Chernobyl and the break-down of the cooling system in Fukushima released large quantities of radionuclides that can be transported across the globe (Parache et al., 2011; Shozugawa et al., 2012; Won et al. 2014; Sayed et al., 2016). Although in the natural environment, the accumulation rate of gamma radiation was observed to be not more than 1 Gy per year, Rhee et al. (2012) reported that it can be accumulated by nuclear accidents at high doses (> 1 Gy) in short periods of time.

Radionuclides have led to a global expansion of nuclear power industries (IAEA, 2007; Reinardy et al., 2011). The generation of radionuclides from stable atoms initially began in laboratory experiments in the 20th century and has

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subsequently grown to an industrial scale. Today many countries obtain about 50% of their energy from nuclear reactors (IAEA, 2007). Nuclear tests (underground, underwater and atmospheric) undertaken during the development of new modern technologies have led to localized and widespread radionuclide contamination (Templeton, 1980; Noshkin et al., 1997). Elevated levels of radionuclides have been reported in organisms in close proximity to fuel processing plants and discharge sites. Radionuclides released into the environment by human activities ultimately end up in the aquatic ecosystems. A radioactive isotope/radioisotope is an isotope of an element that has an inherent nuclear instability. As its nucleus decomposes, it becomes more stable by releasing energy in the form of ionising radiation as particles (alpha radiation, beta radiation or neutrons) or as electromagnetic radiation (X-rays or gamma-rays).

Radioactivity was discovered by the end of 19th century. Radioactive materials in mining areas contain enhanced concentrations of radioactive materials which has led to significant levels of radioactive pollution in the vicinity of mining areas (Vandenhove et al., 2006; Reinardy et al., 2011). Later investigation led to the development of nuclear reactors and nuclear explosive devices. Testing of the nuclear devices generate large quantity of radioactive waste products, such as tritium, carbon-14, caesium-137, strontium-90, iodine-131 and americium-241. Atmospheric detonation of nuclear weapons and major industrial accidents are the largest contributors of radioactive pollution.

Over the years, nuclear accidents have contributed to the large amount of radioactive release. The releases from such events may constitute small to very large activities and the scattering may vary from local to global, depending on the nature of the accident and the environmental conditions of that time. In

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particular significance to the environment are accidents representing different scenarios of release viz. accidental releases of material from functioning nuclear facilities, including accidental releases of cooling water or stored radioactive material into the environment. The releases are most commonly gaseous or liquid, lost through unintentional venting or via waste water. These are typically of lower radioactivity compared to some other accidents, though releases can be of high concentrations in localized areas with the potential for significant environmental impact in the immediate area/surroundings.

Pollution by very high concentrations of radioactive material includes vehicle crashes containing nuclear fuel or nuclear weapons, such as bomber crashes in Palomares (1966) and Thule (1968). Despite significant efforts to reclaim radioactive material, these incidents resulted in the loss of several kilograms of weapons-grade plutonium and/or uranium, the vast majority of which might have remained in the immediate vicinity (<10 km) of the accident site. This resulted in the exposure of local population of biota to high doses of radiation. Further dispersal may occur gradually over time as the material degrades or is mechanically weathered *in situ*.

Other important sources of radioactive pollution include releases from nuclear facilities through catastrophic failure, including explosions or nuclear meltdown. Such incidents typically involve the emission of very large quantities of radioactive material, of a range of radioisotopes, over large geographical regions or even globally. Such incidents include explosions, fires of waste storage (Mayak, 1957; Windscale, 1957; Tomsk-7, 1993), meltdowns and resultant explosions of nuclear reactors (Three Mile Island, 1979; Chernobyl, 1986; Fukushima, 2011). Naturally the immediate area around the accident site is

the most contaminated and contains more radioactive particulate matter. The Chernobyl disaster, the largest accidental release of radiation, demonstrably impacted the local environment spreading to several kilometres. As a result, several adverse genetic and morphological impacts have been observed in many species of plants and animals (Geras'kin et al., 2008; Jaeschke et al., 2011). Such releases can result in elevated concentrations of radionuclides not only in the local plants and animals but even in biota thousands of kilometres away and may persist for several decades (RIFE, 2010).

In addition to accidental release, there are other continuous, far smaller release of radionuclides into the environment from military, energy generation, research and medical industries, predominantly in gaseous and liquid form. These releases are made up of a limited number of radionuclides, frequently with short half-lives that may persist in the environment for only a few days, weeks or a few years. Relatively high concentrations of liquid wastes are permitted to be released into aquatic environments. By and large, dispersal and accumulation data only exists for the predominant isotopes released from nuclear industry (Copplestone et al., 2001; RIFE, 2010; Jaeschke and Bradshaw, 2013). The vast majority of the monitoring activity of concentrations in the aquatic environment, understandably, occurs around the sites/regions where radionuclides are released; however, very little testing occurs in some areas considered not to be affected. However, based upon minimal experimental evidence and typically considering only the major physical and geochemical processes affecting fate and distribution of radionuclides, biological function and interaction have largely been ignored. There has been insufficient research into the connectivity of aquatic environments regarding the dispersal of radioisotopes. The very limited literature

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on radioecology in the tropical regions revolves almost exclusively around the aftermath of nuclear weapons development and testing (Donaldson et al., 1997; Noshkin et al., 1997 Reinardyet al., 2011), an exposure scenario very different from that of routine, permitted releases.

1.5. Toxic effects of ionizing radiation:

1.5.1. Biological effects of direct exposure to ionizing radiation:

Ionizing radiation can affect a variety of processes in exposed cells. It can induce many changes such as DNA damage, changes in gene expression, cell cycle arrest and apoptotic cell death (Jeggo and Lobrich, 2006; Rodemann and Blaese, 2007; Valerie et al*.,* 2007; Anbumani and Mohankumar, 2012; Rhee et al., 2012; Sayed et al., 2014; Azimian et al., 2015; Mohamed et al., 2016; Anbumani and Mohankumar, 2016; Sayed and Mitani, 2016; Sayed et al., 2017). The most important is that ionizing radiation is a potent agent capable of inducing DNA damage such as cross linking, nucleotide base damage and single and double strand breaks (Little, 2000; Huang et al., 2003). If this damage is not correctly restored, it may cause deleterious genetic changes such as mutations and chromosomal aberrations at the initial sites of damage that are further expressed in descendants of irradiated cells. The accumulation of DNA damage caused by ionizing radiation in conjunction with disrupted cellular regulation processes can lead to carcinogenesis (Barcellos-Hoff, 2005; Sowa et al*.,* 2006).

1.5.2 Biological effects of indirect exposure to ionizing radiation:

Indirect damage occurs when ionizing radiation interacts with water molecules in the vicinity of DNA producing free radicals that in turn diffuse short distances to reach the DNA molecule and lead to DNA damage. It is thought that low and high Linear Energy Transfer (LET) radiation, such as γ-

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rays, mainly act on DNA indirectly through the production of radicals. Ionized water (H_2O^+) reacts with other water molecules (H_2O) surrounding it resulting in the formation of the bonds in the DNA double helix, causing DNA damage. Hydroxyl radicals are thought to play a major role in ionizing radiation-induced DNA damage (Morgan and Sowa, 2005).

1.5.2.1. Radiation-induced genomic instability:

Traditionally, the central dogma of radiation biology states that the effects of ionizing radiation are restricted to cells that are hit directly. This paradigm has been challenged by numerous observations in which cells that were not directly traversed by ionizing radiation exhibited responses similar to those of the directly irradiated cells (Kovalchuk and Baulch, 2008). These responses were demonstrated in the cells that were descendants of directly irradiated cells and were termed 'radiation-induced genomic instability' (Hendry, 2001; Morgan, 2003a, b, c; Morgan and Sowa, 2005; Morgan and Sowa, 2007; Kovalchuk and Baulch, 2008).

Genomic instability is characterized by an increased rate of acquisition of alterations in the genome. It occurs in irradiated cells at a delayed time after irradiation, as well as in the progeny of irradiated cells for several generations after exposure (Morgan et al., 1996; Wright, 1998; Little, 2000). Ionizing radiation induced genomic instability manifests itself as an induction of chromosomal aberrations, aneuploidy, gene mutations, gene amplifications, microsatellite instability and cell death (Huang et al., 2003; Morgan, 2003a, b; Suzuki et al., 2003). Radiation-induced genomic instability also includes the formation of micronuclei. These are formed from the acentric chromosome

fragments/lagging chromosome which are not incorporated into the nucleus during cell division (Muller et al., 2004; Hamasaki et al., 2007).
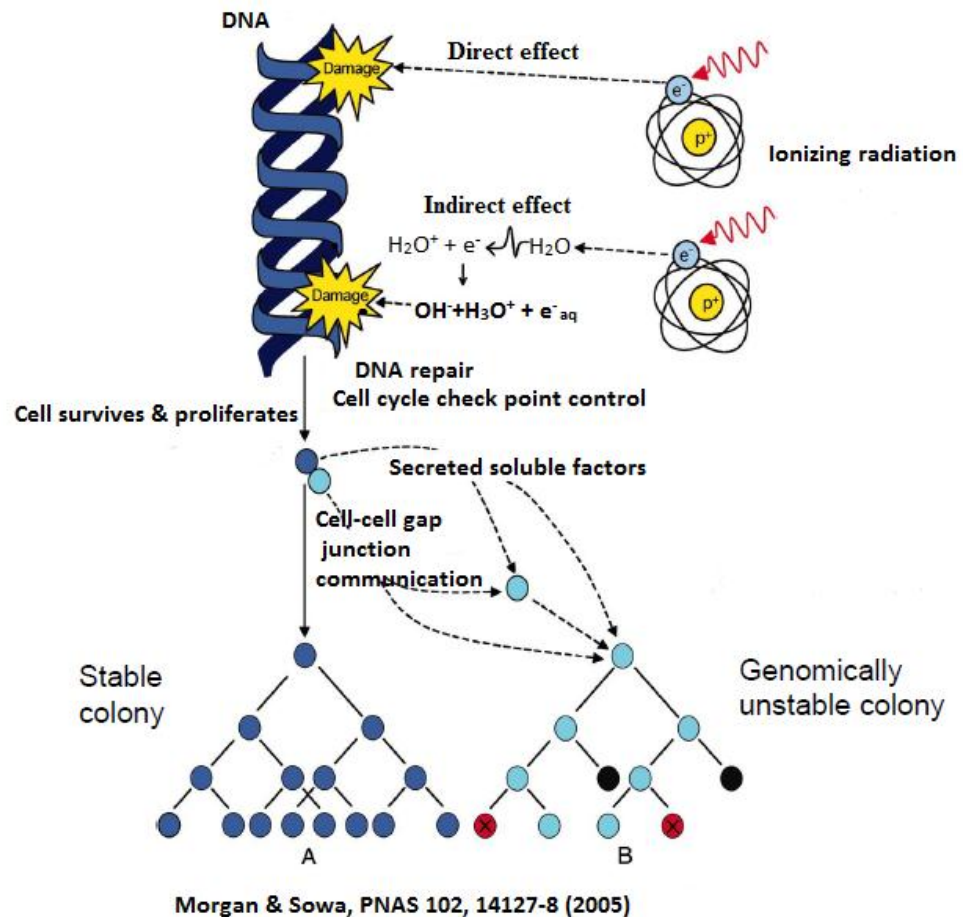
Figure 1.2: Mechanisms of radiation-induced DNA damage: the direct and indirect actions of ionizing radiation. Ionizing radiation can affect DNA both indirectly, where photons ionize water to form hydroxyl radical's .OH, and directly.

Many signaling pathways are involved in the initiation and perpetuation of genomic instability (Kaplan et al., 1997; Limoli et al., 1997). The relative contribution of different pathways depends upon the genetic background of an irradiated cell or organism (Watson et al., 1997). All these molecular changes may be causative factors in cancer development, thereby making the induction of genome instability an important prerequisite to cancer formation and a feature of

cancer (Nowell, 1976; Coleman and Tsongalis, 1999; Goldberg, 2003; Sowa et al., 2006).

1.5.2.2. Radiation induced bystander effects:

Ionizing radiation effects can also be seen in naive cells that were in contact with directly irradiated cells or in naive cells that received certain irradiation 'distress' signals from the cells that had been directly exposed via a growth medium. This communication of exposure is termed as the 'bystander effect' (BE) (Zhou et al., 2000; Morgan 2003a, b; Mothersill and Seymour, 2003; Mothersill and Seymour, 2004; Morgan and Sowa, 2005; Mothersill and Seymour, 2006).

Bystander effects encompass a wide range of genetic alterations, including gross genome rearrangements, chromosome aberrations, sister chromatid exchanges, deletions, duplications, gene mutations and amplifications (Zhou et al., 2000; Huo et al., 2001; Zhou et al., 2002; Klokov et al., 2004; Lorimore et al., 2005; Han et al., 2007; Hamada et al., 2007). They influence gene expression, cellular proliferation, senescence and cell death (Sawant et al., 2001; Sawant et al., 2002; Liu et al., 2006) and are believed to be linked to radiation-induced genome instability (Morgan, 2003c; Huang et al., 2007; Kovalchuk and Baulch, 2008).

Ionizing radiation detaches electrons from atoms or molecules, inducing a charge which may break bonds in the latter. In organisms which are mostly composed of water, the primary mode of action of radiation is the ionization of water into reactive oxygen species (ROS), but can also ionize biological molecules directly, causing damage such as strand breakage in DNA. If not scavenged by antioxidants, reactive oxygen species will cause damage of cellular structures through oxidation, inducing toxic effects. Amongst higher doses of

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radiation, toxicity responds linearly with dose: larger doses give greater effects, more often (NCRP, 1991; Hall and Giaccia, 2006). Using the assumption of the linear no-threshold model, low doses are still expected to produce the same albeit proportionately smaller biological effects of radiation as acute exposures (NCRP, 1991). Several described phenomena indicated the responses may be increased at low doses: such as (i) low dose hypersensitivity, where a disproportionate response is induced/expressed; (ii) adaptive responses, where a detoxifying/reparatory response is stimulated by irradiation; (iii) hormesis, where low doses are beneficial; (iv) non-targeted effects such as the bystander-effect, where irradiated cells emit signals that induce a response in unirradiated cells (Mothersill and Seymour, 2012). An indirect response to the induction of ROS is the expression of antioxidant enzymes; stimulation or adaptation of defence or repair mechanisms may result in a dose where no significant effect occurs (NCRP, 1991; Parsons, 2003). The impacts of low doses of radiation at the population level are not thoroughly understood. Identifying direct responses at the population level, to a radioactive exposure is very difficult or impossible (Woodhead, 2003), therefore it is likely necessary to extrapolate the impacts at organismal or sub-organismal level to those of the population. It would be incorrect to assume that populations are more resistant to the impacts of radiation than an individual; doses sufficient to impact fertility and fecundity are typically non-fatal. The degree of protection required to prevent significant impact on a population may in fact be more than required to prevent a significant effect in an individual. While very low doses of radiation may not observably impact an organism's health, development, fertility or fecundity, genetic damage in the gametes may adversely affect a population. Radiation induced mutation in the DNA or genome can cause genetic instability that can be passed on to the

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progeny (Kadhim et al., 1992; Prise, 2006; Karotki and Baverstock, 2012). This instability can transfer recessive or deleterious genes to offspring and over long periods of time may lead to a higher frequency in the species genome (Trabalka and Allen, 1977; Hertel-Aas et al., 2007).

1.6. Protection of the environment from ionizing radiation:

Although the impact of ionizing radiation on humans through direct/indirect pathways are relatively well understood and documented, the impact of radiation on a wide range of organisms viz. non-human biota including plants and animals are yet to be understood because of great variation in their life cycle, life span and exposure pathway. In addition to this, the quantum of various aspects such as linear energy transfer, relative biological effectiveness and radiation weighting factors (RWF) across the whole animal and plant kingdom are not available. This information is essential to quantify the impact of radiation on non-human biota with whom humans have to co-exist in nature eventually (Singhal et al., 2009). Estimation of equivalent dose of radiation for non-human biota is less defined and there is a need for greater investigation of environmentally-relevant dose rates in non-human biota within the environment (IAEA, 1992; Batlle et al., 2011).

Radionuclides released from nuclear fuel cycles can become incorporated in the biogeochemical cycles of freshwater systems, having entered these systems through direct liquid discharges and through secondary processes such as erosion, runoff and groundwater infiltration from landscapes resulting in low level ionizing radiation exposure of non-humans (ICRP, 1991). It has been demonstrated that Ecological Risk Assessment (ERA) approach developed for chemicals could be applied to the protection of the environment from radiation

(Copplestone et al., 2004). One problem in adapting ERA to the case of radioactive substances relates to the lack of data describing effects of chronic exposure to high/low levels of radiation for non-human species (Garnier-Laplace et al., 2004). Several reports and international bodies are grappling with the problems of regulating exposure of biota (Hinton and Brechignac, 2004; ICRP, 2007; IAEA, 2007) and the most fundamental issue is due to the inadequate scientific data concerning low dose exposure effects.

The risks of ionizing radiation to non-human biota are of considerable current interest, and both the International Commission on Radiological Protection (ICRP) and the International Atomic Energy Agency (IAEA), among others, have on going activities in this area (Chambers et al., 2006; Anbumani and Mohankumar, 2012). Early considerations of the environment in regulation of radioactive releases stating that if releases are controlled enough to protect humans, then the environment will likely be protected were dismissive (ICRP, 1977). This statement, while at the time admitted to be based on little experimental evidence and to be used as a working hypothesis, was widely taken by the scientific community as a statement of fact (Thompson, 1988; Smith, 2005). In recent years, considerable international efforts have been undertaken to develop a regulatory framework for the protection of our environment from the effects of ionizing radiation. Two innovative EC projects were started in 2000: FASSET (Framework for Assessment of Environmental Impact) and EPIC (Environmental Protection from Ionizing Contaminants in the Arctic) with the general aim to develop a methodology for protecting non-human organisms from ionizing contaminants (EPIC Project, 2001; FASSET Project, 2001).

However, continued research, growing awareness of environmental vulnerability and societal pressure have led to a re-evaluation of policies to identify the environment as a distinct entity for protection (IAEA, 2003; ICRP, 2003; Oughton, 2003; ICRP, 2007; Pentreath, 2009; Bréchignac et al., 2011). Generally, the ethical consensus is that the majority of non-human species do not require the same level of protection and also that protecting the individual would be over-conservative. The IAEA (2003) defines its objective of environmental protection as "…to safeguard the environment by preventing or reducing the frequency of effects likely to cause early mortality or reduced reproductive success in individual fauna and flora to a level where they would have a negligible impact on conservation of species, maintenance of biodiversity, or the health and status of natural habitats or communities". However, the ecosystems affected by radionuclide release are diverse and dynamic, with a range of abiotic factors and conditions, and different abundances and diversity of species, all of which are continuously changing. This makes developing a practicable protocol for protection very difficult. The extrapolation of small-scale effects to community and ecosystem level is not yet understood (Copplestone et al., 2004). Hence, until there is sufficient understanding of how exposures (particularly chronic, low-dose exposures) impact populations of organisms, the state-of-the-art relies upon the study of effects in a selected few species and then extrapolation to higher levels of organization. Species selected for study can be (i) reference organisms, chosen as typical or representative of a contaminated environment to demonstrate a range of sensitivities (IAEA, 2003; Pentreath, 2005); (ii) critical species, those that are at most risk because they are the most sensitive, rare or endangered (IAEA, 1999), the actions to ensure the protection of which will indirectly ensure others will be protected; and (iii) sentinel species,

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selected for their role as keystones within their ecosystem, significant impacts upon which will have knock-on effects on the ecosystem itself (Copplestone et al., 2004).

The historically anthropocentric focus of protection is such that research into accumulation and toxicity in organisms in the aquatic environment is concentrated upon a relatively few regions or specific species relevant as a pathway of exposure to humans (Thompson, 1988; Copplestone et al., 2004; ICRP, 2007; RIFE, 2010), ignoring many ecologically important elements. Many early studies simply compared the relative sensitivities of aquatic organisms with the better established mammalian models despite clear disparities in phylogeny, life-history and environment (NCRP, 1991). The test used for mammals is inappropriate for many aquatic organisms as environmental conditions, metabolic rate which is linked to DNA repair and other repair mechanisms and life-span of marine species can vary significantly (NCRP, 1991). The first step to demonstrate protection is to ascertain as to as what level of exposure that populations are experiencing by developing accurate dose estimates. This will also demonstrate which communities are likely the most at risk and thus where the most prominent effects may be found. For this, the activity concentrations of radiation experienced by an organism in the wild must be calculated, including the concentration of radionuclides within the body (internal irradiation) and in the immediate surroundings of the organism.

1.7. Genetic toxicology:

Genetic toxicology evolved from the initial studies of gene mutability demonstrated first by Muller (1927) using X-ray radiation. It investigates the interaction of chemical and physical agents with genetic material, in relation to

subsequent adverse effects. In other words, it identifies and analyses the action of agents with toxicity directed towards the hereditary components of living systems. It can be widely employed for testing the impact of various toxic agents found in the environment, whose presence may alter the integrity of gene pool of wide range of organisms including human beings. These techniques can also be used for the detection and the mechanistic understanding of carcinogenic agents viz. physical and chemical agents. In most of the developing countries including India, pollution of water bodies due to industrial as well as urban discharges is of great concern. Aquatic organisms are threatened due to water pollution resulting from the lack of regulatory framework and their effective implementation. Toxic pollutants interact with genetic material of aquatic organisms leading to a variety of ill effects such as clastogenesis, mutagenesis, tumorigenesis, altered protein expression, reduced fertility and fecundity, which adversely affect the fish species. Biomarkers are rapidly becoming valuable eco-toxicological tools in identifying and evaluating contaminants, their toxicity and bioavailability to aquatic organisms. These biomarkers are highly useful as early warnings for the presence of xenobiotics and their biological effects, which make it possible to implement corrective measures before organisms, communities or ecosystem, suffer irreversible damages. Variety of biomarkers such as chromosomal aberrations test, sister chromatid exchanges, micronucleus assay, comet assay, random amplified polymorphic DNA and ultra-thin isoelectric focusing of proteins are currently used for the assessment of DNA damage. These biomarker studies carried out in fishes are shown to be useful for evaluating the genotoxicity induced by a wide variety of chemicals *in vitro* or *in vivo* exposure of aquatic organisms.

1.8. Model organisms:

The aquatic environment plays a vital role in the functioning of the ecosystem and is intimately related with human health. The increasing human population and industrial development have resulted in increased disposal of anthropogenic chemicals and wastes into the aquatic environment. A majority of these contaminants contain potentially genotoxic and carcinogenic substances. Agents that produce alterations in the nucleic acids and associated compounds at sub-toxic exposure levels resulting in modified hereditary characteristics or DNA inactivation are classified as genotoxins. These are responsible for DNA damage in a variety of aquatic organisms including fishes causing malignancies, reduced growth, abnormal development and reduced survival of embryos, larvae and adults. Genotoxicity not only reduces the 'fitness' (i.e. growth, fertility and fecundity) in wild fish populations, but also pose a risk to human health via the food chain (Al-Sabti and Metcalfe, 1995).

Fish has plenty of advantages which make it as an ideal animal model in the ecotoxicological studies. These include the following (i) it is a very sensitive bioindicator of water quality; (ii) it can highlight the potential danger of new chemicals introduced in the aquatic environment; (iii) it also respond to toxicants in a manner similar to higher vertebrates (Klingerman, 1982). Fishes play an important role in the trophic web, such as bioaccumulation of environmental pollutants and biotransformation of xenobiotics through cytochrome p450-dependent oxidative metabolism like mammals, besides they respond to mutagens at low concentrations. Fish cells retain important traits of fish including poikilothermic behavior, unique xenobiotic metabolism and low rate of repair mechanism. In addition, as compared to mammalian cells, they have been

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shown to be more sensitive for induction of genetic damage. Further, DNA repair has been reported to be slower in fishes than mammals. Thus they can be used as sentinel organisms for bio-monitoring studies.

In the present study we have selected common carp and zebrafish. Common carp *(Cyprinus carpio L.)* belonging to the family Cyprinidae was selected for the study mainly because i. use as a sensitive indicator of environmental stress (Tilak et al., 1981). ii. economically important as human food sources iii. act as 'sentinel' organisms. iv. found in freshwaters such as lakes and rivers. v. its availability in Goa throughout the year. Zebrafish (*Danio rerio*), also belonging to family Cyprinidae, is a freshwater fish native to the Himalayan region.

Some of the advantages of zebrafish as model organisms:

i. Genetic similarity to humans

Zebrafish, being vertebrates, share a high degree of sequence and functional homology with mammals including humans. Due to the conservation of cellular, biological and developmental processes across all vertebrates, studies in fish can give great insight into human disease processes.

ii. Easier to house and care for than rodents

Due to their small size and the relatively simple nature of their natural environment, it is easier to keep zebrafish in what appear to be more natural conditions than it is possible to simulate for mammals. This minimizes housing stress and the impact such stress may have on the outcome of experiments. Not only does this add to the refinement of animal usage, it also minimizes the number of animals that need to be used because it reduces the variation between subjects that can be caused by stress.

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iii. Transparency

Zebrafish embryos and larvae are completely transparent. Hence, it is possible to view the impact of a genetic manipulation or pharmacological treatment using non-invasive imaging techniques. Non-invasive techniques minimize animal suffering. Invasive procedures not only affect the welfare of the animal, but may also affect the experimental outcome. The transparent nature of zebrafish larvae could also mean that the results are more accurate and easier to reproduce.

iv. Lots of offspring

Ensuring a ready supply of animals for research is also easier with zebrafish. Zebrafish produce a much larger number of offspring in each generation than rodents. Rodents have 5-10 offspring per pairing, in comparison to the 200-300 obtained from fish. Zebrafish exhibit a very rapid process of embryonic and larval development into adult.

v. Easier to introduce genetic changes

Zebrafish embryos readily absorb chemicals from their surroundings. These are susceptible to chemical induced DNA damage. Zebrafish are able to withstand much higher levels of chemical mutagens than can be tolerated by rodents. Hence, it is possible to induce a much higher density of mutations in their genome.

1.9. Biomarkers of radiation induced genotoxicity:

Genotoxins produce alterations in the genetic materials at non-lethal, non-cytotoxic concentrations and the changes in the genetic material generally represent the first step ('initiation') of the process of mutagenesis and carcinogenesis. These changes in the genetic material of organisms can be detected at specific levels by using various genotoxicity assays such as

chromosomal aberrations test, sister chromatid exchange, micronucleus assay, comet assay and random amplified polymorphic DNA (RAPD) that have different end points. These assays can detect relatively greater damage to genetic material manifested at chromosomal and DNA levels. Different parameters are used to study the effect of physical agents (radiation) on the cells.

The genotoxic effects of physical and chemical agents/pollutants can be monitored using a broad range of both *in vivo* and *in vitro* biomarker assays. The use of "biomarkers" in aquatic organisms has become a major tool for biomonitoring and assessing the environmental quality. This is based on the responses at the molecular and cellular level which represent the earliest signals of environmental disturbance. Cytogenetic endpoints are proven as sensitive early biomarkers (Dallas and Jha, 2015). Micronucleus (MN) test and comet assays are well-known genotoxicity tests often employed to predict mutagenicity/carcinogenicity of chemicals, including pharmaceuticals under *in vivo* and *in vitro* conditions (Shyama et al. 1991; Tice et al. 2000; Kirkland et al. 2005; Anbumani and Mohankumar 2012; Kadam et al. 2013; Dasgupta et al. 2014; Guilherme et al. 2014; Pandey et al. 2014; Osman 2014; Praveen Kumar et al. 2015). These parameters are routinely used as biomarkers for monitoring aquatic pollution by genotoxic contaminants and can be combined with other physiological and biochemical biomarkers to fully assess the pollution status of various water bodies.

Biomarkers specific to ionizing radiation in representative non-humans biota such as fishes are largely unexplored in the context of radiation protection of the environment. Hence, there is an urgent need to conduct systematic studies using sensitive biomarkers to assess doses of gamma radiation that can cause significant DNA damage in representative non-human biota. Cytogenetic

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endpoints are proven, sensitive early biomarkers of radiation exposure and are useful to monitor environmentally relevant low dose exposures that may occur as a result of unintentional radionuclide discharges. Cytogenetic biomarkers play a key role in assessing the impact of pollutants in apparently healthy sentinel aquatic organisms such as fishes (Anbumani and Mohankumar, 2011). Various cytogenetic techniques like chromosome aberration assay, erythrocyte micronucleus assay, sister chromatid exchanges and comet assay are routinely used to monitor the effects of pollutants in the aquatic ecosystem (Nagpure et al., 2007; Osman, 2014).

1.9.1. Micronucleus assay:

The micronucleus (MN) assay is a simple and sensitive assay to evaluate the genotoxic properties of various agents. Schmid initially studied the formation of micronuclei in the bone marrow cells of Chinese hamsters. Since then it is considered as the quickest methodology for screening the genotoxic agents in various mammalian models (Schmid, 1975). Micronuclei are also known as Howell-Jolly bodies in mammals. Like mammalian species, the MN assay is being adopted to study genotoxicity in fishes, as a measure of clastogenic activity. Fish provide a suitable model for monitoring aquatic genotoxicity and water quality because of their ability to metabolize xenobiotics and accumulate pollutants. MN in fish could be smaller in size, because most fish chromosomes are much smaller (e.g. 1/10 to 1/30 of the size of principal nucleus). The formation of micronuclei depends on the rate of proliferation of cells, which in turn depend on fish species and target tissue and also on the kind of pollutants.

1.9.1.1. Basis of MN formation:

MN is formed in the cytoplasm through the following process:

a) In anaphase, acentric chromosomal fragments lag behind when the centric elements move towards the spindle poles. MN arise from acentric chromosomal fragments or acentric chromosomes that are not incorporated into daughter nuclei at mitosis because they lack a centromere.

b) The lagging elements are not included in the main nuclei and they give rise to separate smaller nuclei called micronuclei. The nucleoplasmic bridge and binucleated cells provide an additional and complementary measure of chromosome rearrangement. This can be scored together with the micronucleus.

The micronucleus test has been recommended for the safety evaluation of new pharmaceuticals, agro-chemicals and industrial chemicals in various regulatory guidelines worldwide.

1.9.2. Single Cell Gel Electrophoresis:

Studies have shown that exposure to genotoxic agents lead to loss of DNA integrity especially DNA strand breakage, which can be used as a sensitive indicator of genotoxicity. Therefore techniques that can detect DNA strand breaks in individual cells are gaining importance (Sigh et al., 1995).

Over the past decade, the comet assay, or single-cell gel electrophoresis (SCGE) has become one of the standard methods for assessing DNA damage with applications in genotoxicity testing, human biomonitoring and molecular epidemiology, ecogenotoxicology as well as fundamental research in DNA damage and repair. The assay attracts adherents by its simplicity, sensitivity, versatility, speed, and economy. The number of publications it spawns rises each year and can also tell us not just how much damage is present in cells, but what form it takes. Although it is essentially a method for measuring DNA breaks, the

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introduction of lesion-specific endonucleases allows detection of, for example, ultraviolet (UV)-induced pyrimidine dimers, oxidized bases and alkylation damage.

The comet assay, involves lysis with detergent and high salt after embedding cells in agarose so that the DNA is immobilized for subsequent electrophoresis. The first demonstration of "comets" was by Östling and Johanson who described the tails in terms of DNA with relaxed supercoiling. Essentially, the comet tail seems to be simply a halo of relaxed loops pulled to one side by the electrophoretic field. It needs to be clearly understood that although the most common variant now employed is alkaline SCGE, a high pH is not essential to detect single-strand breaks. The comet assay is most commonly applied to animal cells, whether in culture or isolated from the organism (e.g., lymphocytes separated from blood or cells from disaggregated tissues).

The procedure of Östling and Johanson (1984) was not widely adopted. A few years later, two research groups independently developed procedures involving treatment at high pH. Singh et al. (1988) lysed cells at pH 10 with 2.5M NaCl, Triton X-100, and Sarkosyl for 1 h, following this with a treatment with alkali (0.3M NaOH) and electrophoresis at the resulting high pH (>13). The protocol introduced by Singh et al. (1988) over time has been simplified. It is now common practice to embed cells in a single layer of agarose on a plain glass slide pre-coated with agarose and dried (in the original method, the cells were in the middle of three agarose layers on a frosted glass slide).

DNA is visualized by fluorescence microscopy after staining with a DNA-binding dye. Ethidium bromide (EB) is probably most commonly used, followed by 4,6-diamidino-2-phenylindole (DAPI). EB is an intercalating dye that binds more efficiently to double-stranded DNA than to single stranded

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DNA. DAPI binds to the major groove, and fluorescence should therefore be dependent on double-stranded structure. DNA strand separation occurs during alkaline treatment, and even if super coiled intact loops of DNA in the head renature readily on neutralization, at least the DNA in the tail should be thoroughly single stranded.

1.9.2.1. Applications of comet assay:

i. Genotoxicity Testing: The comet assay has achieved the status of a standard test in the battery of tests used to assess the safety of novel pharmaceuticals or other chemicals. It is readily applied to *in vivo* experiments, tissues that can be disaggregated to single-cell suspensions, as well as white blood cells. The assay is normally used in its simple form to measure DNA strand breaks, but increased sensitivity, as well as additional information on mechanisms of action, would accrue from inclusion of repair endonucleases to measure specific types of lesions. Genotoxicity is also assessed in cell culture systems, on their own or in conjunction with the microsomal "S9" fraction from liver that provides enzymes to metabolize chemicals to more reactive forms. Chemo protection is also being studied using comet assay. The comet assay is eminently suitable for assessing the ability of phytochemicals, for example, to protect cells against genotoxic insult.

ii. Ecological Monitoring: Suitable organisms can be used in combination with the comet assay as biosensors for the detection of contamination of the environment with genotoxins. Although this work is at an early stage, promising results have been reported. Mussels are popular organisms for assessing contamination in the marine environment. Earthworm coelomocytes have also been used successfully to detect genotoxic compounds in soil. Small rodents living around waste sites have been shown to have elevated

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levels of DNA damage in lymphocytes compared with animals living on clean land (Osman, 2014).

1.9.3. Gene expression:

The effect of the genotoxicants on fish at the gene level can be analyzed by gene expression studies. The measurement of the level of gene expression upon exposure to a test agent can provide information about the mechanism of action of this toxicant and form a sort of genetic signature from the pattern of gene expression. The level of gene expression is studied by analyzing the up or down regulations in the amounts of mRNA transcribed for specific genes. Biological effects elicited by environmental pollutants can be detected at various levels. Early effects will be found at molecular level, as this is one of the first targets in the cell (Moore, 2002). Thus, one approach to detect such radiation effects at an early stage is to study gene expression level which is referred to as toxicogenomics (Weber et al., 2013; Wirbisky et al., 2014). The measurement of gene expression levels after exposure to a mutagen/chemical/physical agent can both provide information about the mechanism of action of toxicant as well as form a "genetic signature" from the pattern of gene expression (Jaafar et al., 2013; Freeman et al., 2014).

2.0. Hypothesis:

Based on the evidence from literature, we hypothesize that determination of radiation-induced DNA damage in non-human biota has potential for risk

assessment. As radiation induces DNA damage, there is a need for systematic studies using sensitive biomarkers to assess the lowest radiation dose that can cause significant DNA damage in representative non-human species. Further, there is a need for evaluating inter-relationship between genotoxicity and reproductive success. This phenomenon has not been fully explored. We predict that ionizing radiation responses are correlated with levels of DNA damage accumulation. The ionizing radiation effects *in vivo* need to be established and determined in different types of tissues. We believe that the ionizing radiation effects are distinct in individuals.

The following objectives were proposed to test this hypothesis:

Objective 1: To evaluate the genotoxic effect of gamma radiation on the somatic cells of Common carp and Zebrafish

Objective 2: To evaluate the toxic effects of gamma radiation on Zebrafish embryo

Objective 3: To evaluate the gamma radiation induced differential expression of selected genes on the somatic cells of Zebrafish and zebrafish embryo

Several experiments were designed to test the proposed hypothesis. The objectives are further described as chapters of this thesis as follows:

Chapter 2: Genotoxic effect of gamma radiation on the somatic cells of fish

The second chapter deals with the genotoxic effect of gamma radiation on fishes. Common carp and zebrafish were irradiated with different doses of acute gamma radiation and the genotoxic effect on the somatic cells of common carp and zebrafish were evaluated using the alkaline single cell gel electrophoresis (SCGE)/Comet assay and the micronucleus (MN) test. MN test can detect the double strand breaks whereas alkaline comet assay can detect the single strand breaks in the cell.

Chapter 3: Toxic effects of gamma radiation on zebrafish embryos

The third chapter deals with the toxic effects of gamma radiation on zebrafish embryos. Zebrafish embryos were irradiated with different doses of gamma radiation and the toxic effects were assessed using various developmental toxicity endpoints viz. mortality rate, hatching rate, morphological malformations, body length and DNA single strand breaks.

Chapter 4: Gamma radiation induced gene expression in zebrafish and embryos

The fourth chapter deals with the gamma radiation induced differential expression of selected genes on the somatic cells of zebrafish adults and embryos. Zebrafish and embryos were irradiated with acute gamma radiation and the changes in expression of key genes involved in DNA repair and developmental genes were evaluated.

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2.1. Introduction:

Living organisms are often being exposed to ionizing radiations in their environment, either by natural sources or man-made sources. The anthropogenic sources of radiation mainly include nuclear power generation, nuclear weapons testing and use, nuclear power plants and use of radiation in medical tools. Radiations of various kinds widely used across the globe for diverse purposes are one of the major pollutants of our water bodies and are toxic to the aquatic biota. Water bodies may get contaminated either regularly by industrial effluents or accidentally from nuclear facilities such as Chernobyl nuclear accident and the recent Fukushima disasters. These are the wake-up calls for employing radiation protection / safety for humans and the environment (Dallas et al., 2012). The toxicity risks of ionizing radiation to non-human biota being of considerable interest to both the International Commission on Radiological Protection (ICRP) and the International Atomic Energy Agency (IAEA), have strongly recommended the impact assessment of radiation on non-human biota (IAEA, 1992; ICRP, 2007). Further, the major objective of the two international agencies, viz. EPIC (Environmental Protection from Ionizing Contaminants in the Arctic) and FASSET (Framework for Assessment of Environmental Impact) is to develop methodologies for protecting non-human biota from ionizing contaminants (EPIC Project, 2001; FASSET Project, 2001).

The FASSET project clearly identifies the need to gather scientific information, in order to:

➢ Identify a range of dose rates at which different degrees of effects in the environment would be expected (including the threshold dose rates at which effects would be expected to be minimal) with a high degree of confidence

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➢ Derive dose rate/response relationships for the chosen endpoints

➢ Determine dose-rate thresholds or minimum dose rates at which effects in the environment are expected to be minimal with a high degree of certainty

➢ Help define the reference organisms for dosimetric purposes

➢ Describe the biological effects of irradiation that are likely to be of significance for protection, at the intended biological level, in an environmental context; and,

➢ Identify reference organisms which can be used in a radiation protection framework (FASSET Project, 2001).

Effects of radiation on biological systems have been studied for several years and it is well known that it induces DNA damage. DNA molecules are the primary target of ionizing radiation within the cell and biological effects of radiation originate mostly from DNA damage (Lemos et al., 2017). Radiation can induce DNA damage by changing the chemical structure either directly or indirectly and these induced DNA damages have been identified mainly as single strand and double strand breaks (Harrison 2013). These damages are not repaired by natural DNA repairing mechanisms and can lead to gene mutation, chromosomal abnormalities and cell death. It is generally accepted that there is no safe dose of radiation and any amount increases the risk of damage (Mothersill and Seymour, 2011; Duport et al., 2012). A linear threshold model has therefore been assumed for low dose radiation, stating that the risk of damage is directly proportional to the exposure dose (Lemos et al., 2017). Experimental studies *in vivo* and *in vitro* allows working with populations with low individual variability and testing a wide range of doses of radiation under controlled condition which are valuable alternative to assess the biological effects of radiation. Further, *in*

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vitro systems can react differently to radiation when compared to the *in vivo* systems (Jarvis and Knowles, 2003; Lemos et al., 2017). The experiments with laboratory animals can provide us more precise insight into the effect of radiation and their underlying mechanisms. Hence, there is a need to conduct systematic studies using sensitive biomarkers to assess precisely the lowest radiation dose that can cause significant DNA damage in representative non-human species.

2.1.1. Model organism:

Fish are often used as biological indicators to detect the presence of genotoxic agents in aquatic environments. Any change in the composition of the environment is an important indicator of water born toxicants (Ayoola, 2008). Generally fish constitutes one of the major sources of cheap nutrition for human beings. The nutritional value of fish depends on the quality of biochemical composition of its body including protein, carbohydrate, fat etc. Protein, in addition to being a major intake energy source, is also a major form in which energy is stored. Even a small change in total body water can cause serious problems in cellular function, principally because of changes in solute concentration. These effects are related to the architecture of cells of which an organism is composed. Fish erythrocytes are sensitive to DNA damage induced by physical or chemical toxicants as they are a major site for the production of ROS due to their role in the oxygen transport via haemoglobin (Anbumani and Mohankumar, 2012). Owing to the direct and continuous contact with the aquatic environment, fish gills which are the organs for respiratory gas exchange, osmoregulation, excretion of nitrogenous waste products and acid base regulation, are directly affected by contaminants. Muscle and gill tissue are the major direct target sites of physical exposure by contaminants in the aquatic environment (Kaweewat and Hofer, 1997; Sharma et al., 2005). Hence, they are

very sensitive to physical and chemical alterations of the aquatic medium. Fish muscle has many functions which aid in swimming and energy production.

2.1.2. Genotoxic biomarkers:

Genotoxicity biomarkers play a key role in assessing the impact of pollutants in apparently healthy sentinel aquatic organisms such as fish (Anbumani and Mohankumar 2011, 2012). Different cytogenetic parameters such as the micronucleus test, chromosome aberration assay and sister chromatid exchanges are regularly used to screen the effects of contaminants in the water system (Osman, 2014). The genotoxic effects of physical and chemical agents can be monitored using a broad range of both *in vivo* and *in vitro* biomarker assays. Cytogenetic endpoints are proven as sensitive early biomarkers (Dallas and Jha, 2015). Micronucleus (MN) test and Comet assays are well known genotoxicity tests often employed to predict mutagenicity/carcinogenicity of chemicals, including pharmaceuticals under *in vivo* and *in vitro* conditions (Anbumani and Mohankumar, 2012; Dasgupta et al., 2014; Guilherme et al., 2014; Pandey et al., 2014; Osman, 2014). The micronucleus assay (MN assay) is a simple and sensitive assay for *in vivo* evaluation of the genotoxic properties of various agents. Micronuclei are the smaller nuclei as compared to the main nucleus of the cell and are formed as a result of chromosomal breakage or spindle damage. A micronucleus (MN) is formed during the metaphase/anaphase transition of mitosis (cell division) (Fenech et al., 2011). The micronucleus assay mainly detects the double strand breaks.

The comet assay is increasingly being used to detect DNA damage in cells. Any cell population may be used to evaluate DNA damage in this assay. Alkaline comet assay can detect the single strand breaks in the cell. Cells with

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increased DNA damage display increased migration and the resulting "comets" are viewed under fluorescence microscopy using appropriate staining. The tail length or tail moment (tail length multiplied by the fraction of DNA in the tail) are used to report the amount of DNA damage (Kumaravel and Jha 2006).

2.1.3. Genotoxic effect of radiation:

Bioaccumulation of radiocesium, induction of DNA damage (Sugg et al., 1996) and increased frequency of MN (Ilyinskikh et al., 1998) were reported in natural populations of fishes collected from two different radiation polluted sites. However, limited reports are available on the toxicity studies in fishes exposed to various kinds of radiation and those that are available are mainly dealing with the effects on the reproductive system (Woodhead, 1977; Hyodo-Taguchi, 1980; Shima and Shimada, 1991; Belova et al., 2007; Adam-Guillermin et al., 2012; Musthafaa et al., 2014). Few reports are available on the induction of chromosomal aberrations in fishes by radiation (Mong and Berra, 1979; Suyamaa and Etoh, 1983). Significant increase of micronuclei were observed in the erythrocytes of *Cyprinus carpio* (Gustavino et al., 2001) and in the gill cells of *Oryzias latipes* (Takai et al., 2004a, b) exposed to X-rays. Low-dose gamma radiation induced significant increase of micronuclei in fish cell line, *in vitro* (Cassidy et al., 2007). Nuclear and cytoplasmic abnormalities, as well as, DNA damage and micronuclei were observed in the erythrocytes of the gamma irradiated fish, *Catla catla* (Hamilton) (Anbumani and Mohankumar, 2011, 2012).

The most severe DNA lesions induced by ionizing toxicants are single and double-strand breaks (Collins, 2004). The alkaline single-cell gel electrophoresis assay allows the early detection of single strand DNA breaks which may be

induced by a range of genotoxic agents including radiation (Mayalpa et al., 1998; Praveen Kumar et al., 2014; D'Costa et al., 2017; Lemos et al., 2017). Gamma irradiation is reported to induce DNA damage and cell cycle perturbations in *Catla catla* (Anbumani et al., 2012; Anbumani and Mohankumar, 2015). A dose-dependent DNA damage was observed in zebrafish exposed to ionizing radiation (Knowles, 2002; Jarvis and Knowles, 2003; Lemos et al, 2017). However, very limited efforts have been made to study acute genotoxic effects of gamma radiation on freshwater fish using genotoxic biomarkers (Anbumani and Mohankumar, 2011, 2012; Anbumani et al., 2012; Anbumani and Mohankumar, 2015).

2.2. Materials and methods:

The main objective of the present study was to evaluate the genotoxic effect of gamma radiation on the somatic cells of Common carp and Zebrafish using the alkaline single cell gel electrophoresis (SCGE)/Comet assay and the micronucleus (MN) test.

2.2.1. Experimental specimen:

Two species of fishes viz. common carp and zebrafish were used in the present study (Plate 2.1).

2.2.1.1. Common carp:

Common carp *(Cyprinus carpio L.)* belonging to the family Cyprinidae was selected for the study, mainly because of its availability in Goa throughout the year, economic importance and use as a sensitive indicator of environmental stress (Tilak et al., 1981). This fish is found in freshwaters such as lakes and river in Europe and Asia. The major morphological features of this fishes are: i. head has triangular shape, somewhat compressed body, thick lips, two pairs of barbels at angle of the mouth ii. dorsal fin base is long with 17-22 branched rays and a

strong, toothed spine in front; dorsal fin outline is concave anteriorly. Iii. the anal fin has 6-7 soft rays; posterior edge of 3rd dorsal and anal fin spines with sharp spinules. The lateral line possesses 32 to 38 scales.

2.2.1.1.1. Collections and maintenance of fish:

The fingerlings of Common carps (2 to 2.5 cm) were collected from 'Fish Breeding and Rearing farm' in Keri, Sattari, Goa, transported in oxygenated / aerated polythene bags during the evening hours, brought to the laboratory and were stocked and maintained in tanks (Plate 2.2 and Plate 2.3).

2.2.1.1.2. Acclimatization in aquaria:

Prior to irradiation, fingerlings of length 7 ± 2.0 cm and weight between 8 – 9.5 g were acclimatized to laboratory conditions in glass aquaria for 2 weeks (temp. $26.2 \pm 2.03°C$, 12-h light/dark cycle, pH 7.1 ± 0.25, DO 8.4 ± 1.1 mg/L). These fishes were fed with oil cake. The water was changed once a day to reduce fecal contamination.

2.2.1.2. Zebrafish:

Zebrafish (*Danio rerio*) belonging to the family Cyprinidae is a freshwater fish, native to Himalayan region. The zebrafish is named so because of the five uniform, pigmented, horizontal, blue stripes on the side of the body, which are reminiscent of a zebra's stripes and extend to the end of the caudal fin. Its shape is fusiform and laterally compressed with its mouth directed upwards. The male is torpedo-shaped, with gold stripes between the blue stripes and the female has a larger, whitish belly and silver stripes instead of gold. Adult females exhibit a small genital papilla in front of the anal fin origin. Zebrafish can grow up to 6.4 cm (2.5 in) in length, although it seldom grows larger than 4 cm (1.6 in) in captivity. Its lifespan in captivity is around two to three years.

2.2.1.2.1. Collections and maintenance:

Adult zebra fish *(Danio rerio)* were procured from Aquaculture farm, Margao, Goa. The fish were maintained in glass aquariua of 50 litre capacity fitted with aerators and heaters at 28±1℃ with 14:10 light: dark photoperiod. Water was manually renewed by changing 50% of the total volume once in every week and by refilling the evaporated water every day. Fishes were fed twice daily with live brine shrimps (*Artemia salina*) and commercial fish feed (Plate 2.4).

2.2.1.2.2. Acclimatization in aquaria:

Prior to irradiation, fingerlings of length (4 to 5) cm and weight between 1 − 1.5 g were acclimatized to the laboratory conditions in glass aquaria for 2 weeks. Fishes were fed twice daily with live brine shrimps (*Artemia salina*). The water was changed once a day to reduce fecal contamination.

2.2.2. LD$_{50/30}$ determination and lethality related observations:

Three hundred (300) fish were procured from the acclimatized batch for the LD$_{50/30}$ determination and were segregated into six groups, 50 fish in each group. The first group of fish was mock irradiated and maintained as controls and the next five groups were used for irradiation. They were exposed to various doses of γ radiation viz. 10, 20, 30, 40 and 50 Gy, respectively. Both the control and irradiated fishes were maintained for 30 days, under close observation. During this period, the number of dead fishes in the control and treated groups were recorded once every 12 hours. Apart from the mortality of fishes, their lethality-related behaviors, as well as morphological manifestations of radiation effects, were observed and recorded. The data was then tabulated, and the lethal dose that caused 50% mortality was calculated according to the method of Spearman−Karber employing the following formula:

$$\textbf{LD}_{\textbf{50}} = \text{LD}_{100} - \sum \frac{\text{Mean death X Conc. difference}}{\text{No. of organism per group}}$$

2.2.3. Irradiation:

Irradiation was carried out at a distance/depth of 80 cm from the radiation source. The dose rate was calculated i.e. machine output was measured by secondary standard dosimeter for (15 x 10) cm field size at a depth of 10 cm in water phantom. Backscatter factor was not considered while calculating the dose at 10 cm depth by considering the height of the fishes as negligible as compared to that of the height of the water chamber. The movement of the fishes was considered as ± 0.5cms. Fish were accommodated in a horizontal slide holder in such a way that they get minimum space for movement by placing glass microscope slides in their groves. The slide holder with the fish were covered with nylon mesh and were placed in the experimental chamber filled with water up to 10 cm level. Fishes (10 per group) were exposed to a whole body irradiation (0.2, 0.4, 0.5, 0.6, 0.8 1, 2, 4, 6, 8 and 10 Gy) allowing 0.5 cm deviations at the center of the dose rate (68.38cGy/min) using a gamma radiation source of a Cobalt Teletherapy Unit at Goa Medical College, Goa. Unirradiated fish maintained on par with the experimental fish were used as controls (mock irradiated). Samples were collected at 24, 48 and 72 hours post irradiation of the irradiated fish as well as controls (Plate 2.5).

2.2.4. Sample collection from fish:

i. Blood:

Blood samples were collected from the caudal vein of each of the control and exposed fishes using a hypodermic syringe under dim light at various time

intervals viz. 24, 48 and 72h of exposure. The fish was collected and was placed up on a stable surface and was held in order to prevent muscle twitching. A heparinised syringe with it, pointed towards the head of the fish was gently inserted under the scales at level of vertebral column just below the lateral line on the middle portion of the tail parallel to the outer portion body axis. The needle was held at 45-degree angle from the surface of the tail. The objective was to locate the caudal vein just below the vertebral column. The needle was slightly guided downward until the needle just touched the vertebrae, then needle was slightly pulled back in order to position the needle into the caudal vein. The plunger of the syringe was pulled out partially to create a negative pressure so that when the vein is encountered blood will flow into the syringe. The syringe was held in place and pulled slowly behind on the plunger to provide the suction, until the desired amount was achieved. When the blood was withdrawn the syringe was removed and inverted in order to mix the blood.

ii. Gill and muscle tissues:

The gill and muscle tissues (~50 mg each) were collected from each of the control and exposed fish at various exposure time intervals of 24, 48 and 72h. They were washed twice with chilled phosphate buffer saline (Ca^{2+} Mg^{2+} free) to remove blood cells and transferred to an ice-cold homogenization buffer (1-X Hanks' balanced salt solution, 20 mM EDTA, 10% dimethyl sulphoxide, pH 7.0–7.5). The tissue was then homogenized with a Potter-Elvehjem homogenizer to obtain a single-cell suspension. The homogenate was centrifuged at 3,000 rpm at 4 ºC for five min and the pellet was finally suspended in chilled phosphate buffered saline

Each sample was transferred to a micro centrifuge tube placed on ice in an ice box to prevent endogenous DNA damage occurring during sample preparation and also to inhibit DNA repair in the unfixed cells.

2.2.5. Cell viability assay:

Prior to the MN test and Comet assay, the cell count and the cell viability were checked to ensure that there were a minimum number of living cells to perform the assay. These tests were conducted with the use of a haemocytometer employing trypan blue dye exclusion test. Samples showing more than 90% viability and a cell count of a minimum of 10^6cells/ml were used for the MN test and comet assay.

2.2.6. Procedure of MN test:

➢ 0.1 ml of peripheral blood was drawn in a heparinized syringe by caudal vein. Gill cell collected after homogenate were used.

➢ A thin smear was made on pre cleaned slide with the help of a glass slide

➢ The slides were prepared in clean and dust free environment at room temperature

➢ The smears were fixed by dipping the slides with smear in absolute methanol for 5-10 min

➢ The slides were then dried for at least 1 hour

➢ The slides were then stained in 0.13% May Grunwald's stain for 2-3 minutes

➢ Then the slides were washed with double distilled water (DDW)

➢ Smear were stained in diluted May Grunewald's stain for 3-6 minutes

➢ Slides were washed with DDW and dried

➢ Smear were stained with 6-10% Giemsa stain on phosphate buffer for 30 minutes

➢ Washed in DDW and dried overnight

➢ Slides were made permanent with DPX-mountant and drying it overnight at 60 $^{\circ}$C over hot plate

➢ The slides were then observed under microscope using 40/100X objectives and the micro nucleated cells were scored (Plate 2.6).

2.2.7 Comet assay (Single cell gel electrophoresis):

The protocol of Singh *et al.* (1988) was employed for studying single strand breaks.

2.2.7.1 Slide preparation

1. Base layer first coat: 60 µl normal melting agarose (Himedia- RM 6249 - 100G) (1% in DDW, microwave or heat until near boiling and the agarose dissolves) was smeared on frosted (Fisher Scientific- ½ gross, 3" x 1" x 1mm; Cat no. 12-544-5CY) side of the slide and dried for 20 minutes in room temperature by placing cover glass Himedia- No. 1, thickness 0.13 to 0.16 mm, size- 25 x 50 mm) on the smear. Then cover the glass was slowly removed.

2. Base layer second coat: 200 µl of agarose (1% in PBS, Ca^{2+} and Mg^{2+} free, Himedia- TS 006- 20L) was placed on the first coat and then, covered with cover glass and kept in ice for 5 minutes. Cover glass was later removed.

NOTE: If the gels do not adhere to the slides properly, avoiding humidity and/or increasing the concentration of NMP agarose in the lower layer to 1.5% should eliminate the problem.

3. Second layer: Samples (Whole blood, gill and muscle) (15 µl) with low melting agarose (75 µl) (Himedia-RM861 100G) (low melting, 0.5 % in PBS) in dim light suspension was placed on the base layer. All the steps hereafter are undertaken under dim light.

4. Third layer: Cover glass was removed and 100 μl low melting agarose (0.5% in PBS) was placed over 2nd layer and covered with cover glass and the gel was solidified on ice for 5 minutes.

NOTE: Slides should be labeled prior to dipping.

2.2.7.2. Slide processing

a.   Lysis

5. The slides were placed in 50 ml of cold (4°C) lysing solution for 2 h.

6. After lysis, the slides were removed gently from the lysing solution. Slides were rinsed carefully in Tris for 5 minutes to remove detergents and salts.

b.   Unwinding

7. Fill the buffer reservoirs with freshly made electrophoresis buffer pH >13 until the liquid level completely covers the slides (avoid bubbles over the agarose). Place slides side by side on the horizontal gel box near one end, sliding them as close together as possible.

8. Slides were kept in the alkaline buffer for 20 minutes to allow for unwinding of the DNA.

c.   Electrophoresis

9. Slide with microgel were electrophoresed at 20 Volt 275 mA for 20 minutes (dim light).

NOTE: The goal is to obtain migration among the control cells without it being excessive. The optimal electrophoresis duration differs for different cell types. A lower voltage, amperage and a longer electrophoresis time may allow for increased sensitivity. Different gel boxes will require different voltage settings to correct for the distance between the anode and the cathode.

10. The power was turned off and gently the slides were lifted from the buffer and placed on a drain tray.

d.   Neutralization

11.   Micro-gel slides were immersed in neutralizing solution for 10 minutes and repeated this step for twice.

12.   Dehydrated slides were placed in absolute methanol for 5- 10 minutes and dehydrated in incubator at 50° C for 30 minutes (comet slides can be stored in boxes until use).

e.   Staining

13.   15 μl of ethidium bromide (1x) was placed in equally spaced droplets over the clear area of gel and covered with cover glass.

14.   The slides were screened for analysis of comet images (Plate 2.7).

2.2.7.3. Comet capture and analysis

15. A minimum of two slides were scored at 250x magnification per sample and 25 comets were evaluated per slide using CASP image analysis software with fluorescent microscope having specific filters connected through ProgRes CapturePro V 2.8.0 camera. This computerizing image analysis system (CASP software) acquires images, computes the integrated intensity profiles for each cell, estimates the comet cell components and then evaluates the range of derived parameter (Plate 2.8). The DNA damage was quantified % tail DNA as a parameter employing the following formula was used.

$$\% \text{ Tail DNA} = 100 - \text{Head DNA}$$

2.2.8. Statistical analysis:

Statistical analyses were performed using IBM SPSS 23 software package. Since the data of the comet assay are expressed as percentage (%) values, the data were arcsine transformed and tested for normality and homogeneity using the Shapiro-Wilk test and Levene's test respectively. Data

was analysed with the student's t-test and ANOVA. Within the single experiment, the significance of each dose against their respective control values was evaluated by the student's t-test. Dose-response and the time-response of fishes to gamma irradiation were determined by one-way ANOVA with pairwise Tukey tests to identify differences between specific treatment groups. The dose-response and time-response of the tissues to gamma irradiation in each tissue were determined by a two-way ANOVA, with a post hoc pairwise Tukey test to identify differences between specific treatment groups. A multivariate analysis of variance (MANOVA) was used to test the influence of different variables and their interaction on the DNA damage parameters. A level of probability of $p < 0.05$ was considered as statistically significant data.

2.3. Results:

2.3.1. LD$_{50/30}$ determination and lethality related observations:

Data on the lethality of irradiated fishes, common carp and zebrafish are represented in Table 2.1 and Table 2.2 respectively. Toxicity of gamma radiation was indicated by the morphological and behavioral anomalies, including pigmentation, surfacing movements, excessive mucous production, hemorrhage in the eyes, rapid opercular movement, loss of equilibrium, lateral lying at the bottom of the aquaria and death observed in these irradiated fishes. The LD$_{50/30}$ study was carried out for both the above species of fish and it was found to be 21.6 Gy for common carp and 20.4 Gy for zebrafish.

Table 2.1: Data for LD$_{50/30}$ determination for common carp based on Arithmetic method of Karber

Dose (Gy)	Dose difference	No. of fishes used	No. of alive	No. of dead	Mean death	Mean death dose difference
0	0	50	50	0	0	0
10	10	50	39	11	5.5	55

20	10	50	24	26	18.5	185
30	10	50	14	36	31	310
40	10	50	6	44	40	400
50	10	50	0	50	47	470

$$LD_{50} = LD_{100} - \sum \frac{Mean\ death\ X\ Conc.diff}{No.of\ organism\ per\ group}$$

$$= 50 - \frac{1420}{50}$$

$$= 50 - 28.4$$

$$= 21.6\ Gy\ is\ the\ LD_{50/30}\ for\ common\ carp.$$

Table 2.2: Data for $LD_{50/30}$ determination for zebrafish based on Arithmetic method of Karber

Dose (Gy)	Dose difference	No. of fishes used	No. of alive	No. of dead	Mean death	Mean death dose difference
0	0	50	50	0	0	0
10	10	50	36	14	7	70
20	10	50	23	27	20.5	205
30	10	50	13	37	32	320
40	10	50	3	47	40	400
50	10	50	0	50	48.5	485

$$LD_{50} = LD_{100} - \sum \frac{Mean\ death\ X\ Conc.diff}{No.of\ organism\ per\ group}$$

$$= 50 - \frac{1480}{50}$$

$$= 50 - 29.6$$

$$= 20.4\ Gy\ is\ the\ LD_{50/30}\ for\ zebrafish.$$

2.3.2. Genotoxic study in common carp:

2.3.2.1. Gamma radiation induced micronuclei in common carp blood:

The micronuclei induced in the erythrocytes of common carp by various

doses (0.2, 0.4, 0.5, 0.6, 0.8 1, 1.5, 2, 4, 6, 8 and 10 Gy) of gamma radiation at

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different time intervals (24 h, 48 h and 72 h) are summarized in Table 2.3 and represented graphically in Figure 2.1.

A significant increase of micronuclei ($p < 0.0001$) was induced in all individuals of common carp at doses 0.8 to 10 Gy of gamma radiation in comparison to their respective controls as per the Students t-test. Further, a dose dependent increase of the micronuclei was observed, with a minimum (0.09 ± 0.01) at the lowest dose (0.8 Gy) and the maximum (1.94 ± 0.15) at the highest dose (10 Gy) at 24h of post irradiation. A similar trend was also observed at other intervals, viz. 48h and 72h. One way ANOVA of the data on the dose dependent DNA damage observed in the control and treated animals showed significance (f= 758.1, $p < 0.001$; f= 1440, $p < 0.001$; f= 3589, $p < 0.001$) at all-time intervals (24, 48 and 72 h, respectively).

Time dependent increase of the micronuclei was induced by 0.8 Gy of gamma radiation, the lowest frequency of MN (0.09 ± 0.018) observed at 24h post treatment, which increased to 1.00 ± 0.026 at 48 h and reached the maximum (1.10 ± 0.023) by 72 h. A similar trend was observed for the other doses too, i.e (1, 1.5, 2, 4, 6, 8 and 10 Gy). One way ANOVA of the DNA damage observed at different time intervals showed significant decreases in DNA damage with the a $P<0.001$ (f= 36.98, $p < 0.001$; f= 81.47, $p < 0.001$; f= 202.7, $p < 0.001$; f= 345.4, $p < 0.001$; f= 290.4, $p < 0.001$; f= 1308, $p < 0.001$; f= 939.5, $p < 0.001$) for all doses studied (1, 1.5 2, 4, 6, 8 and 10 Gy, respectively). Further, dose and time dependency was assessed by employing Two-way analysis of variance. A significant difference was observed between doses (F= 5604; p< 0.0001) and time (F= 3738; p< 0.0001). The NOEL dose of 0.6 Gy was found in which we didn't observe any significant level of damage in erythrocytes of common carp according to micronucleus parameter.

Table 2.3: MN frequency (%) in erythrocytes of common carp exposed to different doses of gamma radiation at different time intervals.
*p< 0.05, **p< 0.01 and ***p< 0.001

	24 h	48 h	72 h
Control	0.06±0.015	0.07±0.016	0.06±0.016
0.2 Gy	0.06±0.012	0.06±0.011	0.07±0.014
0.4 Gy	0.06±0.016	0.07±0.016	0.06±0.016
0.5 Gy	0.07±0.022	0.06±0.017	0.06±0.016
0.6 Gy	0.07±0.022	0.08±0.020	0.07±0.021
0.8 Gy	0.09±0.018*	0.10±0.026**	0.11±0.023**
1 Gy	0.10±0.021**	0.18±0.020***	0.15±0.022***
1.5 Gy	0.10±0.021**	0.23±0.037***	0.28±0.037***
2 Gy	0.12±0.024**	0.56±0.040***	1.36±0.040***
4 Gy	0.36±0.110***	1.12±0.130***	1.90±0.150****
6 Gy	1.04±0.110***	1.94±0.190***	3.06±0.240***
8 Gy	1.48±0.130***	2.40±0.150***	4.98±0.190****
10 Gy	1.94±0.158***	3.32±0.162***	6.08±0.181***

Figure. 2.1: Frequency of MN (%) in erythrocytes of common carp exposed to various doses of gamma radiation at 24, 48 and 72 h time intervals

*p< 0.05, **p< 0.01 and ***p< 0.001

Molecular cytogenetic studies on the toxic effects of gamma radiation in Zebrafish and Common carp

2.3.2.2. Gamma radiation induced DNA damage (% of tail DNA) in blood cells of common carp:

The DNA damage in the blood cells of gamma irradiated common carp at various dose (0.2, 0.4, 0.5, 0.6, 0.8 1, 1.5, 2, 4, 6, 8 and 10 Gy) in different time intervals (24 h, 48 h and 72 h) are summarized in Table 2.4 and represented graphically in Figure 2.2.

The fish irradiated with doses 0.6-10 Gy of gamma radiation exhibited significant DNA damage ($p<0.001$) compared to their controls. The % tail DNA to increased in a dose-dependent manner, with a minimum (9.29 ± 4.003) at the lowest dose (0.6 Gy) and the maximum (74.90 ± 9.540) at the highest dose (10 Gy) at 24h of post irradiation. A similar trend was observed for the for other time intervals i.e. 48h minimum (5.25 ± 0.661) and maximum (65.51 ± 10.540), for 72h minimum (3.13 ± 1.028) and maximum (46.20 ± 9.120). One-way ANOVA of the data on the dose dependent DNA damage observed in the control and treated animals showed significance (F= 406.9, $p<0.001$; F= 305.9, $p<0.001$; F= 179.5, $p<0.001$) at all the time intervals studied (24, 48 and 72 h respectively). The time-yield study indicated significant DNA damage at all the time intervals and for all doses studied as per the t-test. Fishes irradiated with 0.6 Gy showed the highest DNA damage (9.29 ± 41.003) at 24 h post treatment, which decreased considerably at later times and reached the minimum (3.13 ± 1.028) by 72 h. A similar trend was observed for the other doses too i.e (4, 6, 8 and 10 Gy). One way ANOVA of the DNA damage observed at different time intervals showed significant decrease in the DNA damage with the (f= 117.5, $p<0.001$; f= 122.90, $p<0.001$; f= 104.257, $p<0.001$; f= 220.9, $p<0.001$; f= 108.1, $p<0.001$; f= 35.50, $p<0.001$; f= 57.57, $p<0.001$; f= 61.82, $p<0.001$; f= 23.16, $p<0.001$) for all doses studied (0.6, 0.8, 1, 1.5, 2, 4, 6, 8 and 10 Gy, respectively). Further, dose

and time dependent was assessed by employing Two-way analysis of variance. The doses (f= 873.2; $p< 0.001$) and time (f= 321.0; $p< 0.001$) showed significant difference. The NOEL dose of 0.5 Gy was found in which we didn't observe any significant level of damage in erythrocytes of common carp according to comet assay parameter.

Table 2.4: Percentage of tail DNA (Mean ± SD) in blood cells of common carp exposed to different doses of gamma radiation at different time intervals. **p< 0.01 and ***p< 0.001

	24 h	48 h	72 h
Control	1.21±0.200	1.18±0.320	1.20±0.230
0.2 Gy	1.22±0.220	1.21±0.240	1.20±0.340
0.4Gy	1.20±0.317	1.21±0.343	1.21±0.201
0.5Gy	1.23±0.416	1.22±0.313	1.16±0.310
0.6Gy	9.29±1.003***	5.25±0.661***	3.13±1.028***
0.8Gy	14.76±1.687***	10.13±0.932***	6.12±0.922***
1Gy	20.02±1.972***	18.11±1.721***	10.19±0.986***
1.5Gy	35.54±3.207***	20.10±1.631***	15.12±1.565***
2 Gy	56.55±4.960***	39.67±3.110***	34.05±1.950***
4 Gy	60.48±8.910***	59.82±7.300***	36.18±5.410***
6 Gy	66.80±5.950***	60.10±8.410***	36.20±6.040***
8 Gy	71.19±6.140***	62.40±7.280***	37.90±7.950***
10 Gy	74.90±9.540***	65.51±10.540***	46.20±9.120***

Figure.2.2. Percentage of DNA damage (% tail DNA) in blood cells of common carp exposed to various doses of gamma radiation at 24, 48 and 72 h time intervals
Note: **p< 0.01 and ***p< 0.001

2.3.2.3. Gamma radiation induced micronuclei in common carp gill cells:

The micronuclei in the gill cells of gamma irradiated common carp at various dose (0.2, 0.4, 0.5, 0.6, 0.8 1, 1.5, 2, 4, 6, 8 and 10 Gy) in different time intervals (24 h, 48 h and 72 h) are summarized in Table 2.5 and represented graphically in Figure 2.3.

A significant increase of micronuclei (p<0.0001) was seen in all gamma-irradiated fish irradiated in comparison to their respective controls. The frequency of micronuclei was seen to increase in a dose-dependent manner with a minimum (0.42 ± 0.021) at the lowest dose (0.5 Gy) and maximum (1.96 ± 0.178) at the highest dose (10 Gy) at 24h post irradiation. This dose-dependent increase was also observed at the 48h time interval [minimum 0.59 ± 0.017 at the lowest dose (0.5 Gy)] and the maximum 2.65 ± 0.119 at the highest dose (10 Gy)] as well as for the 72h time interval [minimum 0.64 ± 0.013 at the lowest dose (0.5 Gy) and maximum 3.04 ± 0.140 at the highest dose (10 Gy)]. Additionally, significant dose-dependent variation in the frequency of micronuclei was observed in the control and treated animals at the 24, 48 and 72 h time intervals (F= 591.6, *p*< 0.001; F= 1858, *p*< 0.001; F= 2325, *p*< 0.001 respectively). A significant time-dependent increase of MN was also observed for all the doses (0.5-10 Gy) of gamma radiation. For the 0.5 Gy treatment group, the lowest frequency of MN (0.42 ± 0.021) was observed at 24h post treatment and the maximum frequency of MN was recorded at 72h post treatment (0.64 ± 0.013). This time-dependent increase was observed for all the treatment groups i.e 0.6, 0.8, 1, 1.5, 2, 4, 6, 8 and 10 Gy. The variation in the DNA damage between the time intervals was found to be highly significant for all the doses studied (0.5 Gy: F= 443.8, *p*< 0.001; 0.6 Gy: F= 71.70, *p*< 0.001; 0.8 Gy: F= 380.0, *p*< 0.001; 1 Gy: F= 241.2, *p*< 0.001; 1.5 Gy: F= 105.7, *p*< 0.001; 2 Gy: F= 1199, *p*< 0.001; 4 Gy: F= 2110,

$p < 0.001$; 6 Gy: F= 161.3, $p < 0.001$; 8 Gy: F= 250.4, $p < 0.001$; 10 Gy: F= 137.1, $p < 0.001$). The interactions of dose and time on the frequency of micronuclei by the two-way analysis of variance was found to be highly significant (F= 4235; $p < 0.001$ F= 1656; $p < 0.001$ respectively). The NOEL dose of 0.4 Gy was found in which we didn't observe any significant level of damage in gill cells of common carp according to micronucleus parameter.

Table 2.5: MN frequency in gill cells of common carp exposed to different doses of gamma radiation at different time intervals.
 Note: *p< 0.05, **p< 0.01 and ***p< 0.001

	24 h	48 h	72 h
Control	0.32±0.013	0.32±0.019	0.31±0.011
0.2 Gy	0.32±0.016	0.31±0.013	0.32±0.018
0.4 Gy	0.31±0.013	0.31±0.015	0.32±0.019
0.5 Gy	0.42±0.021	0.59±0.017*	0.64±0.013*
0.6 Gy	0.54±0.024*	0.61±0.025*	0.69±0.034**
0.8 Gy	0.65±0.020*	0.83±0.028***	0.96±0.027***
1 Gy	0.78±0.011***	0.92±0.025***	0.99±0.026***
1.5 Gy	0.82±0.023***	0.95±0.037***	1.02±0.032***
2 Gy	0.98±0.032***	1.20±0.034***	1.70±0.035***
4 Gy	1.03±0.031***	1.50±0.032***	1.95±0.032***
6 Gy	1.35±0.091***	1.84±0.090***	2.04±0.084***
8 Gy	1.50±0.113***	2.05±0.095***	2.56±0.109***
10 Gy	1.96±0.178***	2.65±0.119***	3.04±0.140***
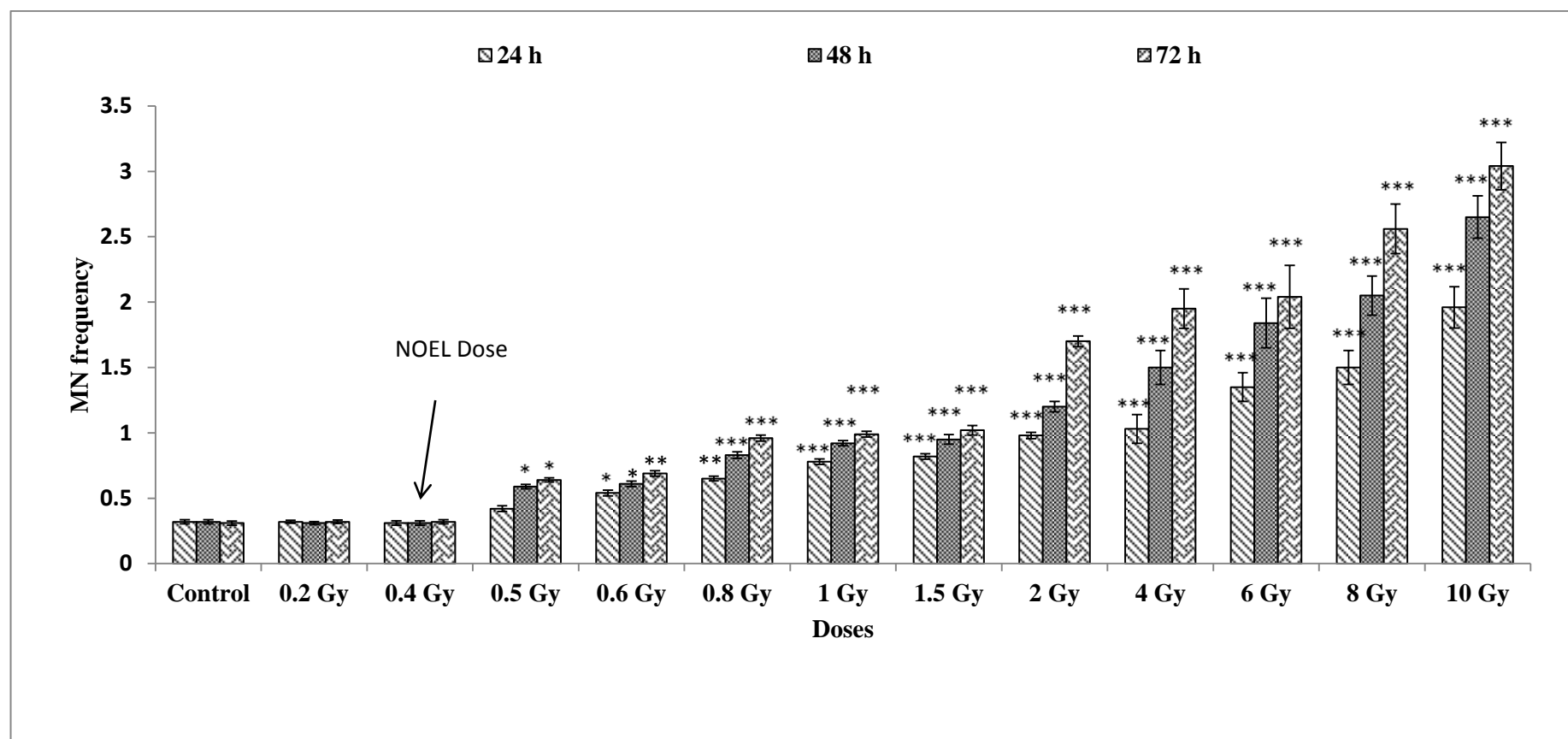
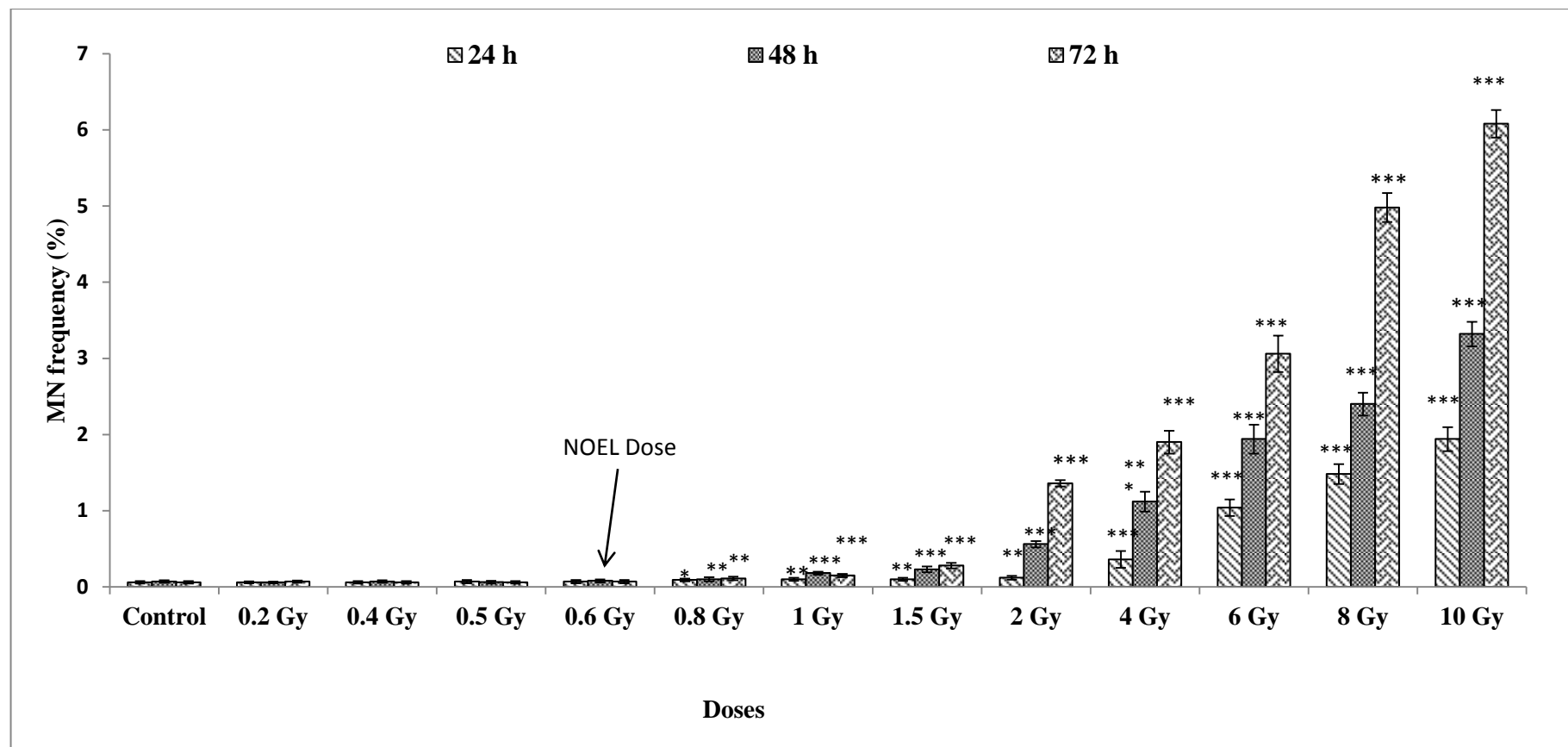**Figure.2.3.** MN frequency in gill cells of common carp exposed to various doses of gamma radiation at 24, 48 and 72 h time intervals
Note: *p< 0.05, **p< 0.01 and ***p< 0.001

Molecular cytogenetic studies on the toxic effects of gamma radiation in Zebrafish and Common carp

2.3.2.4. Gamma radiation induced DNA damage (% tail DNA) in gill cells in common cap:

The DNA damage in the gill cells of gamma irradiated common carp at various dose (0.2, 0.4, 0.5, 0.6, 0.8 1, 1.5, 2, 4, 6, 8 and 10 Gy) in different time intervals (24 h, 48 h and 72 h) are summarized in Table 2.6 and represented graphically in Figure 2.4.

DNA damage was observed to be significant ($p<0.001$) in the gills cells of all the irradiated fishes compared to their respective controls. A significant dose-dependent increase (F = 699.5, $p< 0.001$; F = 451.5, $p< 0.001$; F = 252, $p< 0.001$) of % tail DNA was observed for all the time intervals ie 24h, 48h and 72h respectively. The time-yield study indicated significant DNA damage at all-time intervals and for (0.4-10 Gy) doses studied as per the t-test. Fishes irradiated with 0.5 Gy showed the highest DNA damage (10.35 ± 0.216) at 24h post treatment, which decreased (8.35 ± 0.331) at 48h and reached the minimum (8.05 ± 0.381) by 72 h. A similar trend was observed for the other doses too i.e (0.6, 0.8, 1, 1.5 2, 4, 6, 8 and 10 Gy). One way ANOVA of the DNA damage observed at different time intervals showed significant decrease in the DNA damage with the (f= = 155.6, $p< 0.001$; f= 894.8, $p< 0.001$; f= 2438, $p< 0.001$; f= 1808, $p< 0.001$; f= 1560, $p< 0.001$; f= 133.8, $p< 0.001$; f= 54.01, $p< 0.001$; f= 39.53, $p< 0.001$; f= 30.04, $p< 0.001$; f=16.52, $p< 0.001$) for all doses studied (0.5, 0.6, 0.8, 1, 1.5, 2, 4, 6, 8 and 10 Gy, respectively). Further, dose and time dependent was assessed by employing Two-way analysis of variance. The doses (f= 1297, $p< 0.001$) and time (f= 639.3, $p< 0.001$) showed significant difference. The NOEL dose of 0.4Gy was found in which we didn't observe any significant level of damage in gill cells of common carp according to comet assay parameter.

Table 2.6: Percentage of tail DNA (Mean ± SD) in gill cells of common carp exposed to different doses of gamma radiation at different time intervals.
Note: *p< 0.05, **p< 0.01 and ***p< 0.001

	24h	**48h**	**72h**
Control	6.89±0.120	6.93±0.132	6.92±0.123
0.2 Gy	6.90±0.212	6.94±0.214	6.95±0.234
0.4Gy	8.06±0.232	8.05±0.234	8.05±0.210
0.5Gy	10.35±0.216***	8.35±0.331*	8.05±0.381*
0.6Gy	28.02±0.900***	19.60±0.761***	12.46±0.803***
0.8Gy	60.57±1.187***	51.31±0.917***	29.40±0.950***
1Gy	65.57±1.720***	63.81±1.210***	34.18±0.860***
1.5Gy	68.69±1.207***	58.60±1.310***	38.60±1.157***
2 Gy	71.23±3.960***	63.81±3.200***	46.98±2.950***
4 Gy	75.21±4.910***	60.28±5.130***	52.14±5.061***
6 Gy	76.42±5.950***	62.14±5.410***	52.85±6.504***
8 Gy	78.14±5.140***	62.87±6.280***	56.65±7.500***
10 Gy	80.21±8.540***	65.26±9.400***	57.29±9.200***

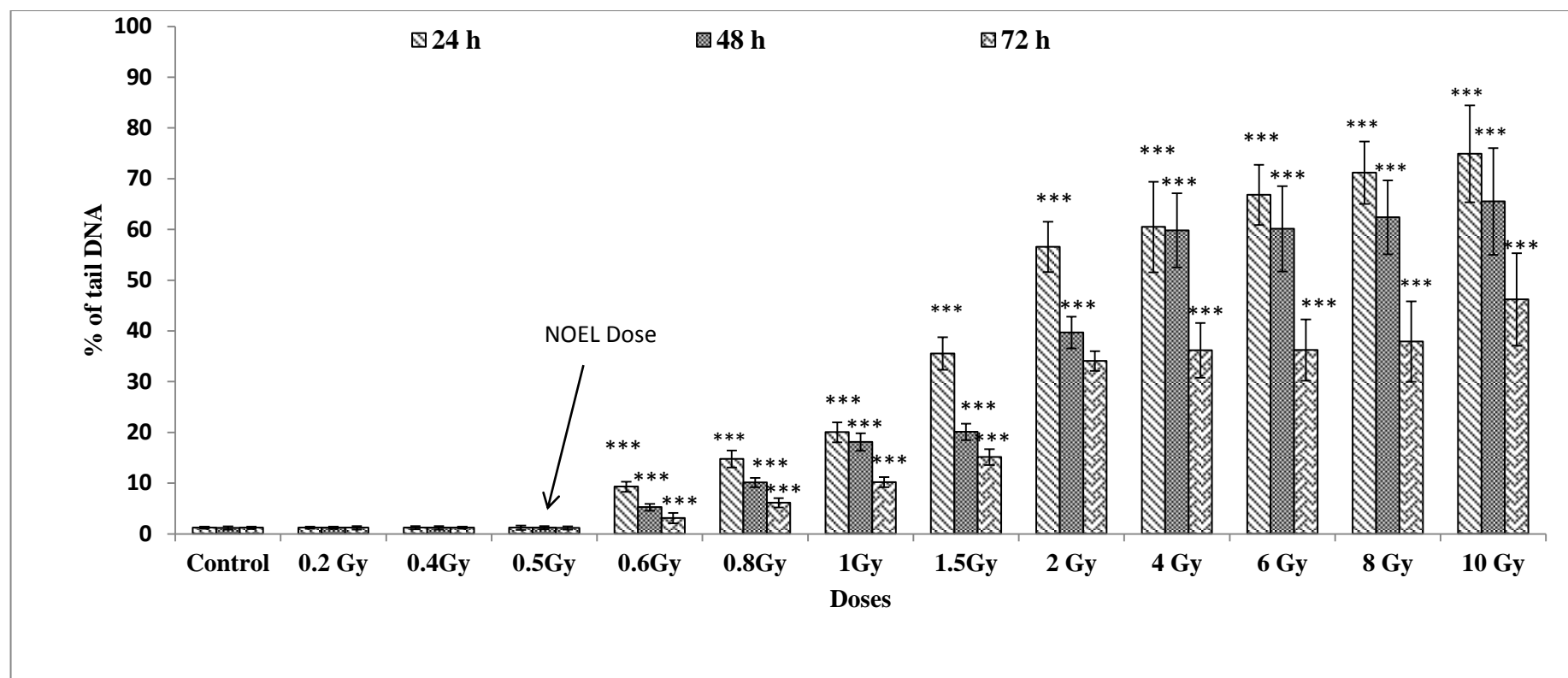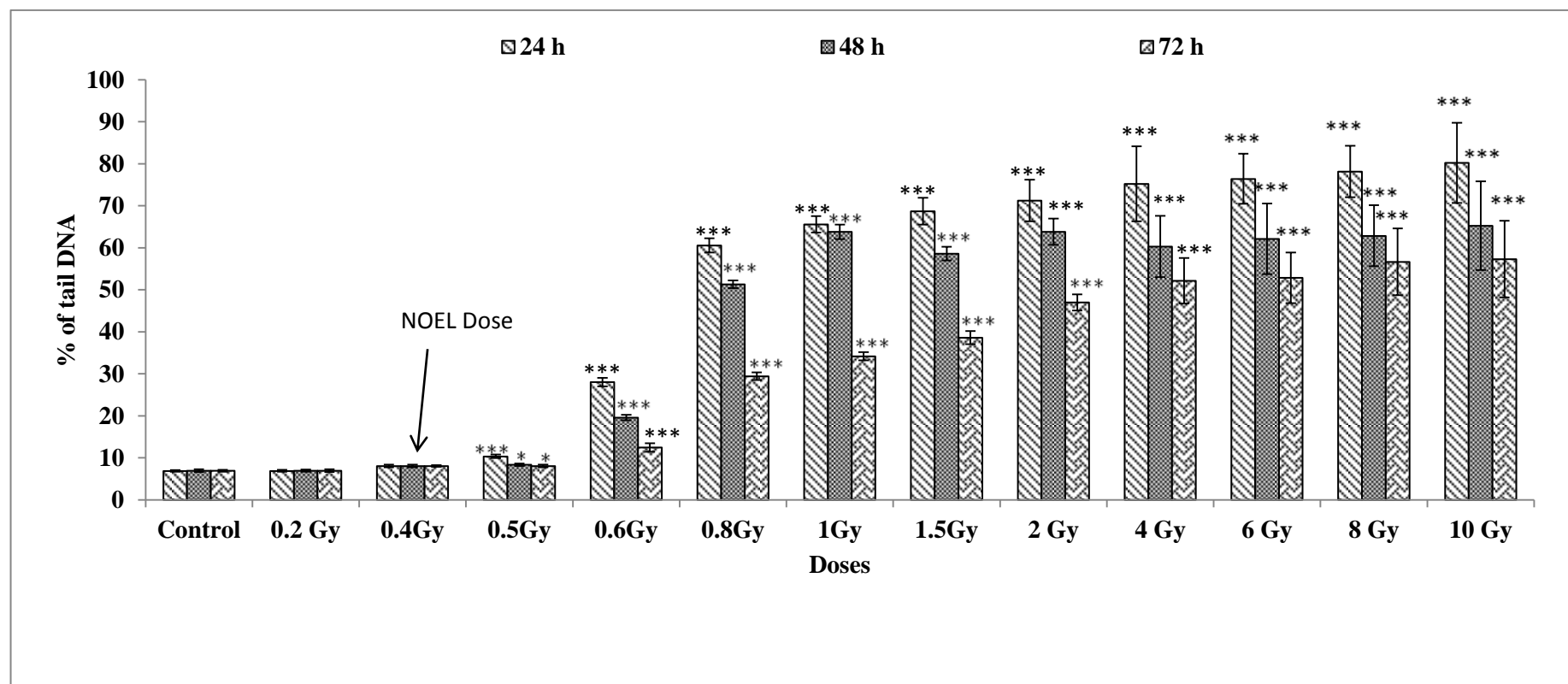**Figure.2.4:** Percentage of DNA damage dose in gill cells of common carp exposed to various doses of gamma radiation at 24, 48 and 72 h time intervals
Note: *p< 0.05, **p< 0.01 and ***p< 0.001

2.3.2.5. Gamma radiation induced DNA damage (% tail DNA) in muscle tissue of common carp:

The DNA damage in the muscle cells of gamma irradiated common carp at various dose (0.2, 0.4, 0.5, 0.6, 0.8 1, 1.5, 2, 4, 6, 8 and 10 Gy) in different time intervals (24 h, 48 h and 72 h) are summarized in Table 2.7 and represented graphically in Figure 2.5.

Significant DNA damage was seen in all the fishes irradiated by various doses (0.5-10 Gy) of gamma radiation in comparison to their respective controls as per the t-test. Further, a significant dose-dependent increase (f = 828.5, $p<$ 0.001) of the mean % tail DNA was observed, with a minimum (21.51 ± 0.231) at the lowest dose (0.5 Gy) and maximum (66.59 ± 7.241) at the highest dose (10 Gy) at 24h post irradiation. A similar trend was observed for the for other time intervals i.e. 48h (f = 479.2, $p<$ 0.001) minimum (12.88 ± 0.250) and maximum (51.35 ± 5.468), for 72h (f = 432.2, $p<$ 0.001) minimum (8.36 ± 0.354) and maximum (36.20 ± 4.171). The time dependent also indicated significant DNA damage at all-time intervals and for (0.5-10 Gy) doses studied as per the t-test. Fishes irradiated with 0.5 Gy showed the highest DNA damage ((21.51 ± 0.231) at 24h post treatment, which decreased (12.88 ± 0.250) at 48h and reached the minimum (8.36 ± 0.354) by 72 h. A similar trend was observed for the other doses too i.e (0.6, 0.8, 1, 1.5 2, 4, 6, 8 and 10 Gy). One way ANOVA of the DNA damage observed at different time intervals showed significant decrease in the DNA damage with the (f= = 5553, $p<$ 0.001; f= 2118, $p<$ 0.001; f= 3402, $p<$ 0.001; f= 1833, $p<$ 0.001; f= 1845, $p<$ 0.001; f= 1998, $p<$ 0.001; f= 389.8, $p<$ 0.001; f= 141.8, $p<$ 0.001; f= 113.1, $p<$ 0.001; f=69.46, $p<$ 0.001) for all doses studied (0.5, 0.6, 0.8, 1, 1.5, 2, 4, 6, 8 and 10 Gy, respectively). Further, dose and time dependent was assessed by employing Two-way analysis of variance. The

doses (f= 1822, *p*< 0.001) and time (f= 1621, *p*< 0.001) showed significant difference. The NOEL dose of 0.4 Gy was found in which we didn't observe any significant level of damage in muscle tissue of common carp according to comet assay parameter.

Table. 2.7: Percentage of tail DNA (Mean ± SD) in muscle tissue of common carp exposed to different doses of gamma radiation at different time intervals. *p< 0.05, **p< 0.01 and ***p< 0.001

	24 h	**48 h**	**72 h**
Control	5.24±0.182	5.42±0.214	5.26±0.111
0.2 Gy	5.26±0.151	5.34±0.180	5.24±0.173
0.4Gy	5.31±0.193	5.30±0.215	5.23±0.160
0.5Gy	21.51±0.231***	12.88±0.250***	8.36±0.354*
0.6Gy	24.78±0.540***	18.15±0.420***	12.36±0.281***
0.8Gy	43.25±0.452***	37.21±0.485***	26.24±0.465***
1Gy	46.35±0.642***	40.16±0.590***	30.21±0.570***
1.5Gy	48.36±0.750***	39.21±0.782***	28.54±0.653***
2 Gy	50.25±0.877***	41.64±0.840***	27.15±0.756***
4 Gy	55.75±1.280***	48.38±2.452***	31.81±1.980***
6 Gy	60.47±3.544***	47.15±2.983***	33.25±4.211***
8 Gy	62.17±3.540***	50.24±5.480***	35.14±2.470***
10 Gy	66.59±7.241***	51.35±5.468***	36.20±4.171***
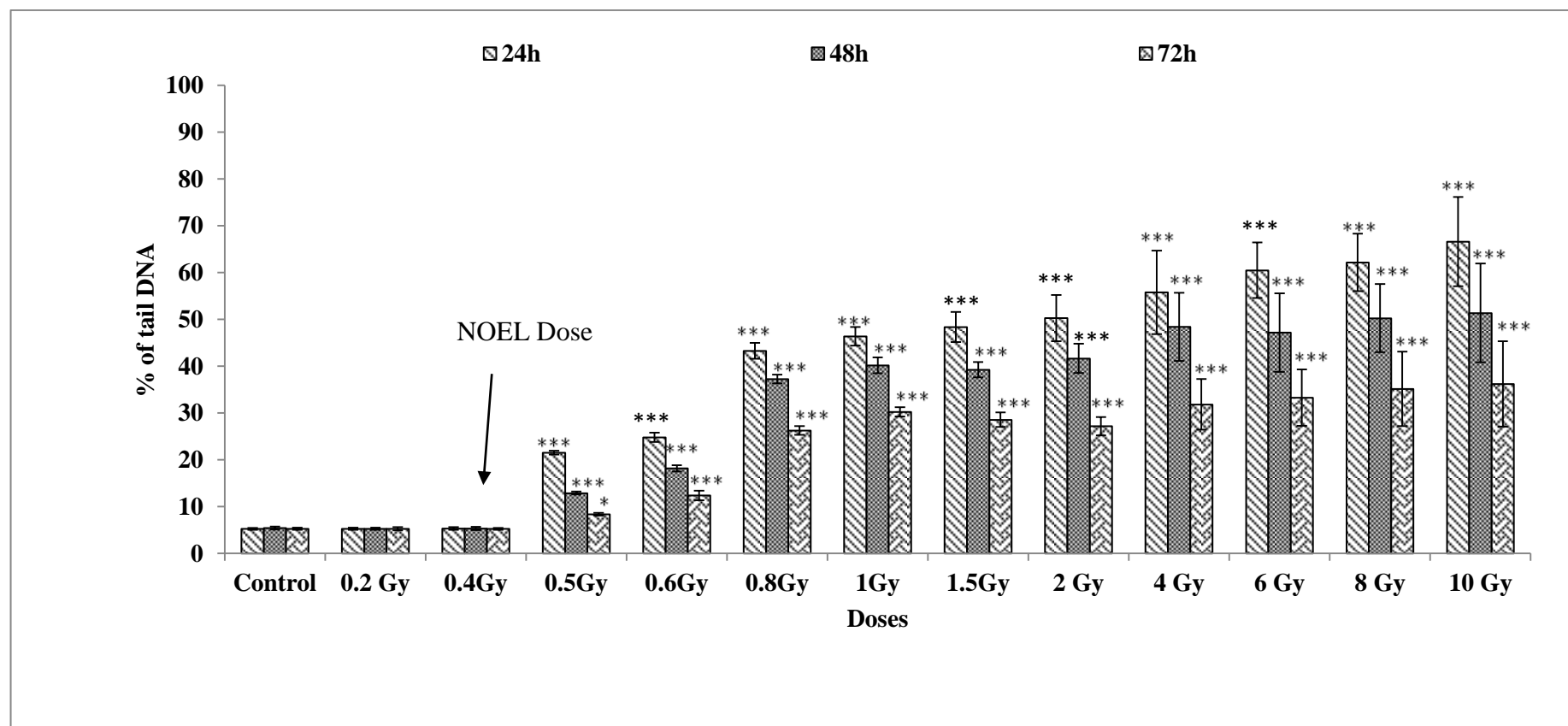
Figure.2.5: Percentage of DNA damage in muscle cells of common carp exposed to various doses of gamma radiation at 24, 48 and 72 h time intervals Note: *p< 0.05, **p< 0.01 and ***p< 0.001

2.3.2.6. Influence of different variables on DNA damage parameter (MN) in common carp exposed to gamma radiation:

Common carp showed significant variation between tissues, time intervals and doses of gamma radiation with respect to the MN test as per the Multivariate analysis of variance (MANOVA) summarized in Table 2.8. The interaction between time and dose (F = 62.015, $p < 0.001$), tissue and time (F = 107.68, p<0.001) and tissue and dose (F = 183.45, $p < 0.001$) influence the induction of MN significantly. The interaction between different variables (Tissue, Time and Dose F = 20.84, $p < 0.001$) was also observed to significantly influence the formation of MN.

Table 2.8: Multivariate analysis of variance (MANOVA) testing the influence of tissue, time and dose on MN in the common carp exposed to gamma radiation

Source	Type III Sum of Squares	df	Mean Square	F	Significance
Time	105.855	2	52.927	736.160	0.001
Dose	1499.268	12	124.939	1737.764	0.001
Tissue	152.717	1	152.717	2124.119	0.001
Time * Dose	107.008	24	4.459	62.015	0.001
Time * Tissue	15.485	2	7.742	107.688	0.001
Dose * Tissue	158.273	12	13.189	183.450	0.001
Time * Dose * Tissue	35.959	24	1.498	20.840	0.001

2.3.2.7. Influence of different variables on DNA damage parameters (% tail DNA) in common carp exposed to gamma radiation:

Significance variation between tissues, time and doses of gamma radiation on DNA damage parameter (% tail DNA) in common carp fish was observed as per Multivariate analysis of variance (MANOVA) it is summarized in Table 2.9. Significant interaction between different variables in % tail DNA (Tissue, Time and Dose F = 6.07, p<0.001). The interaction of tissue and time (F = 3.25, p<0.013), time and dose (F = 43.52, p<0.001), and tissue and dose (F = 96.96, p<0.001).

Table 2.9: Multivariate analysis of variance (MANOVA) testing the influence of tissue, time and dose on % tail DNA in the common carp exposed to gamma radiation

Source	Type III Sum of Squares	df	Mean Square	F	Significance
Time	5753.308	2	2876.654	1256.151	0.001
Tissue	7760.135	2	3880.068	1694.313	0.001
Dose	80725.183	12	6727.099	2937.528	0.001
Time * Tissue	29.791	4	7.448	3.252	0.013
Time * Dose	2392.088	24	99.670	43.523	0.001
Tissue * Dose	5329.552	24	222.065	96.969	0.001
Time * Tissue * Dose	667.708	48	13.911	6.074	0.001

2.3.3. Genotoxic study in Zebrafish:

2.3.3.1. Gamma radiation induced micronuclei in blood cells zebrafish:

The micronuclei in the erythrocytes of gamma irradiated zebrafih at various dose (0.2, 0.4, 0.5, 0.6, 0.8 1, 1.5, 2, 4, 6, 8 and 10 Gy) in different time intervals (24 h, 48 h and 72 h) are summarized in Table 2.10 and represented graphically in Figure 2.6.

A significant increase of micronuclei ($p < 0.0001$) was seen in all individuals of zebrafish irradiated by various doses (0.6 to 10 Gy) of gamma radiation in comparison to their respective controls as per the Students t-test. Further, a dose dependent increase of the micronuclei was observed, with a minimum (0.06± 0.012) at the lowest dose (0.6 Gy) and the maximum (1.32 ± 0.021) at the highest dose (10 Gy) at 24h of post irradiation. A similar trend was also observed for the other intervals 48h with a minimum (0.05± 0.021) at the lowest dose (0.6 Gy) and the maximum (3.24 ± 0.313) at the highest dose (10 Gy) and 72h with a minimum (0.10 ± 0.012) at the lowest dose (0.6 Gy) and the maximum (6.71 ± 0.318) at the highest dose (10 Gy). One way ANOVA of the data on the dose dependent DNA damage observed in the control and treated animals showed significance (f= 166.9, $p < 0.001$; f= 1083, $p < 0.001$; f= 2951, $p < 0.001$) at all-time intervals (24, 48 and 72h, respectively). Time dependent increase of the micronuclei was observed, with at 0.6 Gy, the lowest frequency of MN (0.06± 0.012) was observed at 24h post treatment, which increased (0.05± 0.021) at 48 h and reached the maximum (0.10 ± 0.012) by 72 h. A similar trend was also observed for the other doses, i.e (1, 1.5, 2, 4, 6, 8 and 10 Gy). One way ANOVA of the DNA damage observed at different time intervals showed significant decreases in DNA damage (0.5 Gy: F= 28.81, $p < 0.001$; 0.6 Gy: F= 7073, $p < 0.001$; 0.8 Gy: F= 6028, $p < 0.001$; 1 Gy: F= 13040, $p < 0.001$; 1.5 Gy:

F= 23130, *p*< 0.001; 2 Gy: F= 1555, *p*< 0.001; 4 Gy: F= 1648, *p*< 0.001; 6 Gy:

F= 1001, *p*< 0.001; 8 Gy: F= 911.1, *p*< 0.001; 10 Gy: F =975.2, *p*< 0.001)for all

doses studied (0.5, 0.6, 0.8, 1, 1.5 2, 4, 6, 8 and 10 Gy, respectively). Further,

dose and time dependent was assessed by employing Two-way analysis of

variance. The doses (f= 3600; p< 0.0001) and time (f= 8524; p< 0.0001) showed

significant difference. The NOEL dose of 0.6 Gy was found in which we didn't

observe any significant level of damage in erythrocytes of zebrafish according to

micronucleus parameter.

Table 2.10: MN frequency in erythrocytes of zebrafish exposed to different doses of gamma radiation at different time intervals.
*p< 0.05, **p< 0.01 and ***p< 0.001

	24 h	**48 h**	**72 h**
Control	0.05±0.014	0.06±0.014	0.07±0.012
0.2 Gy	0.05±0.011	0.05±0.014	0.06±0.011
0.4 Gy	0.06±0.016	0.06±0.015	0.07±0.014
0.5 Gy	0.06±0.012	0.06±0.012	0.06±0.016
0.6 Gy	0.06±0.012	0.05±0.021	0.08±0.012
0.8 Gy	0.06±0.015	0.50±0.023	0.98±0.012**
1 Gy	0.07±0.023*	0.10±0.021**	1.05±0.024***
1.5 Gy	0.09±0.023*	0.24±0.024***	1.64±0.024***
2 Gy	0.12±0.021**	0.54±0.034***	3.54±0.054***
4 Gy	0.34±0.191***	2.25±0.173***	5.14±0.215***
6 Gy	0.86±0.211***	2.58±0.129***	6.02±0.254***
8 Gy	1.24±0.213***	3.02±0.215***	6.25±0.319***
10 Gy	1.32±0.216***	3.24±0.313***	6.71±0.318***
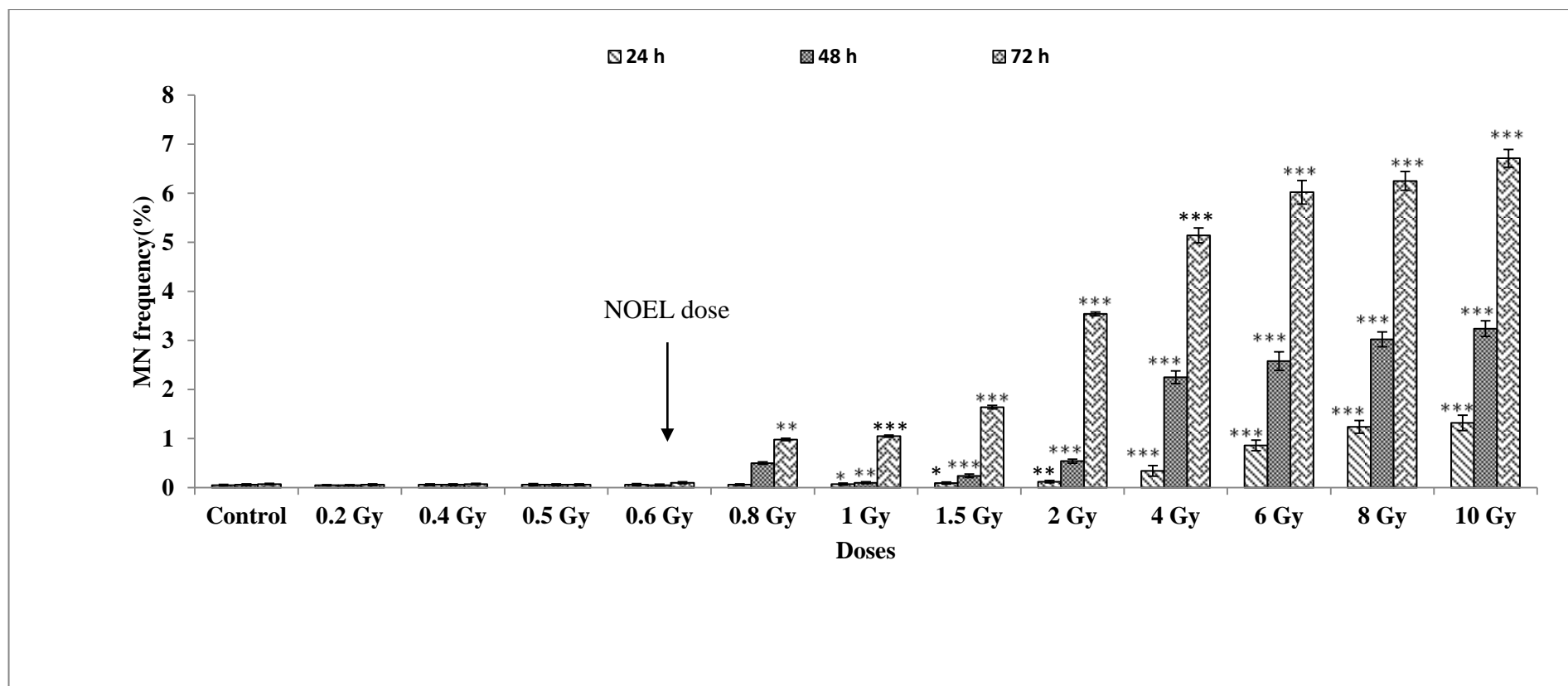
Figure.2.6: MN frequency in in blood cells of zebrafish exposed to various doses of gamma radiation at 24, 48 and 72 h time intervals
Note: *p< 0.05, **p< 0.01 and ***p< 0.001

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2.3.3.2. **Gamma radiation induced DNA damage (% of tail DNA) in blood cells of zebrafish:**

The DNA damage in the erythrocytes of gamma irradiated zebrafish at various dose (0.2, 0.4, 0.5, 0.6, 0.8 1, 1.5, 2, 4, 6, 8 and 10 Gy) in different time intervals (24 h, 48 h and 72 h) are summarized in Table 2.11 and represented graphically in Figure 2.7.

Significant DNA damage (P<0.001) was seen in all the fishes irradiated by various doses (0.6-10 Gy) of gamma radiation in comparison to their respective controls as per the t-test. Further, a dose dependent increase of the mean % tail DNA was observed, with a minimum (8.14 ± 0.900) at the lowest dose (0.6 Gy) and the maximum (58.14 ± 9.400) at the highest dose (10 Gy) at 24h of post irradiation. A similar trend was observed for the for other time intervals i.e. 48h minimum (5.56 ± 0.766) and maximum (49.54 ± 10.400), for 72h minimum (4.54 ± 1.280) and maximum (36.56 ± 9.540). One way ANOVA of the data on the dose dependent DNA damage observed in the control and treated animals showed significance (f= 262.4, *p*< 0.001; f= 163.8, *p*< 0.001; f= 128.2, *p*< 0.001) at all-time intervals studied (24, 48 and 72 h respectively). The time dependent also indicated significant DNA damage at all-time intervals and for all doses studied as per the t-test. Fishes irradiated with 0.6 Gy showed the highest DNA damage (8.14 ± 0.900) at 24h post treatment, which decreased (5.56 ± 0.766) at 48h and reached the minimum (4.54 ± 1.280) by 72 h. A similar trend was observed for the other doses too i.e (0.8, 1, 1.5, 2, 4, 6, 8 and 10 Gy). One way ANOVA of the DNA damage observed at different time intervals showed significant decrease in the DNA damage with the (f= 34.03, *p*< 0.001; f= 57.50, *p*< 0.001; f= 112.2, *p*< 0.001; f= 88.02, *p*< 0.001; f= 39.83, *p*< 0.001; f= 12.42, *p*< 0.001; f= 14.99, *p*< 0.001; f= 21.43, *p*< 0.001; f= 12.31, *p*< 0.001) for all

doses studied (0.6, 0.8, 1, 1.5, 2, 4, 6, 8 and 10 Gy, respectively). Further, dose and time dependent was assessed by employing Two-way analysis of variance. The doses (f= 873.2; *p*< 0.001) and time (f= 321.0; *p*< 0.001) showed significant difference. The NOEL dose of 0.5 Gy was found in which we didn't observe any significant level of damage in erythrocytes of zebrafish according to comet assay parameter.

Table. 2.11: Percentage of Tail DNA (Mean ± SD) in erythrocytes of zebrafish exposed to different doses of gamma radiation at different time intervals
*p< 0.05, **p< 0.01 and ***p< 0.001

	24 h	48 h	72 h
Control	3.02±0.120	4.05±0.232	3.21±0.323
0.2 Gy	3.21±0.212	3.32±0.234	4.01±0.234
0.4Gy	4.12±0.342	3.74±0.314	3.46±0.301
0.5Gy	5.14±0.416	4.65±0.313	4.05±0.313
0.6Gy	8.14±0.900*	5.56±0.766	4.54±1.280
0.8Gy	15.28±1.870**	10.25±0.917**	9.47±0.922**
1Gy	26.21±1.720***	19.54±1.672***	16.43±0.959***
1.5Gy	32.14±3.070***	24.68±1.310***	19.45±1.650***
2 Gy	40.25±4.600***	35.42±3.101***	27.15±1.510***
4 Gy	48.21±8.100***	42.15±6.300***	33.54±5.100***
6 Gy	51.24±5.500***	44.25±8.100**	35.70±5.040***
8 Gy	55.47±4.140***	48.75±7.800***	36.18±7.500***
10 Gy	58.14±9.400***	49.54±10.400***	36.56±9.540***
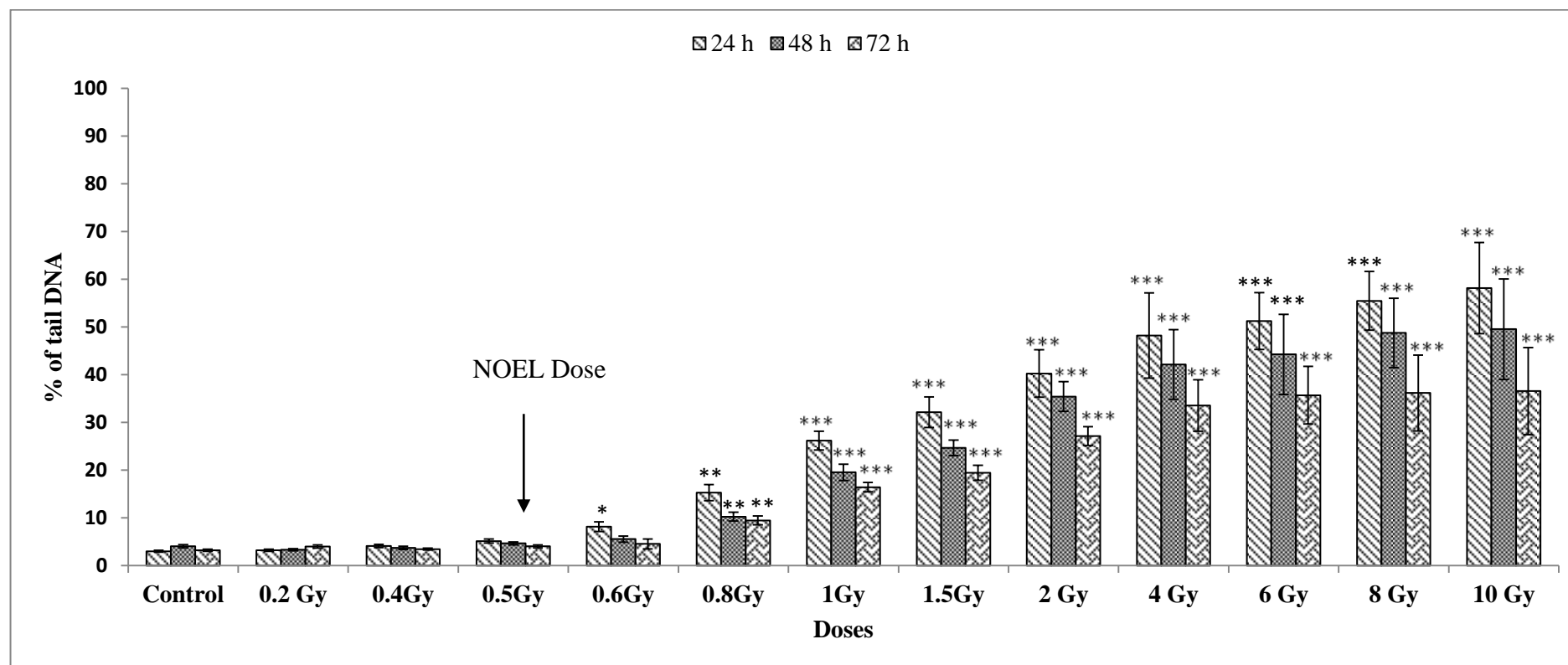
Figure.2.7: Percentage of DNA damage in blood cells of zebrafish exposed to various doses of gamma radiation at 24, 48 and 72 h time intervals
Note: *p< 0.05, **p< 0.01 and ***p< 0.001

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2.3.3.3. Gamma radiation induced micronuclei in gill cells of zebrafish:

The micronuclei in the gill cells of gamma irradiated zebrafish at various dose (0.2, 0.4, 0.5, 0.6, 0.8 1, 1.5, 2, 4, 6, 8 and 10 Gy) in different time intervals (24 h, 48 h and 72 h) are summarized in Table 2.12 and represented graphically in Figure 2.8.

A significant increase of micronuclei was seen in all individuals of zebrafish irradiated by various doses (0.6-10 Gy) of gamma radiation in comparison to their respective controls as per the t-test. Further, a dose dependent increase of the micronuclei was observed, with a minimum (0.44 ± 0.025) at the lowest dose (0.6 Gy) and the maximum (1.02 ± 0.258) at the highest dose (10 Gy) at 24h of post irradiation. Similar trend followed for other time intervals i.e. 48h a minimum (0.47 ± 0.023) at the lowest dose (0.5 Gy) and the maximum (2.14 ± 0.219) and for 72h a minimum (0.46 ± 0.024) at the lowest dose (0.5 Gy) and the maximum (3.54 ± 0.135)   One way ANOVA of the data on the dose dependent DNA damage observed in the control and treated animals showed significance (f=45.71, *p*< 0.001; f= 592, *p*< 0.001; f= 2799, *p*< 0.001) at all the time intervals (24, 48 and 72 h, respectively). Time dependent significant number of MN was observed at all the time intervals and for various doses (0.6-10 Gy) of gamma radiation studied as per the t-test. At 0.6 Gy, the lowest frequency of MN (0.44 ± 0.025) was observed at 24h post treatment, which increased considerably at the later time intervals and reached the maximum (0.46 ± 0.024) by 72 h. A similar trend was also observed for the other doses, i.e (0.6, 0.8, 1, 1.5, 2, 4, 6, 8 and 10 Gy). One way ANOVA of the DNA damage observed at different time intervals showed significant decreases in DNA damage with the (f= 4.06, *p*< 0.05; f= 586.6, *p*< 0.001; f= 368.4, *p*< 0.001 f= 467, *p*< 0.001; f= 443.8, *p*< 0.001; f= 621.5, *p*< 0.001 f= 341.4, *p*< 0.001; f= 790.1, *p*< 0.001; f= 360.3) for all doses

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studied (0.6, 0.8, 1, 1.5, 2, 4, 6, 8 and 10 Gy, respectively). Further, dose and time dependent was assessed by employing Two-way analysis of variance. The doses (f= 1861; *p*< 0.001) and time (f= 2157; *p*< 0.001) showed significant difference. The NOEL dose of 0.6 Gy was found in which we didn't observe any significant level of damage in gill cells of zebrafish according to micronucleus parameter.

Table. 2.12: MN frequency in gill cells of zebrafish exposed to different doses of gamma radiation at different time intervals
 Note: *p< 0.05, **p< 0.01 and ***p< 0.001

|	**24 h**	**48 h**	**72 h**
Control	0.42±0.011	0.38±0.010	0.41±0.013
0.2 Gy	0.41±0.010	0.41±0.012	0.42±0.011
0.4 Gy	0.42±0.013	0.43±0.014	0.39±0.016
0.5 Gy	0.45±0.023	0.44±0.015	0.47±0.020
0.6 Gy	0.44±0.025	0.47±0.023	0.46±0.024
0.8 Gy	0.47±0.016	0.50±0.016**	0.72±0.021**
1 Gy	0.51±0.022*	0.67±0.021**	0.78±0.024***
1.5 Gy	0.54±0.028**	0.74±0.024***	0.92±0.031***
2 Gy	0.57±0.032***	0.85±0.035***	1.05±0.041***
4 Gy	0.60±0.051***	1.05±0.061***	1.48±0.055***
6 Gy	0.72±0.101***	1.54±0.109***	2.01±0.124***
8 Gy	0.90±0.153***	1.93±0.115***	3.11±0.099***
10 Gy	1.02±0.258***	2.14±0.219***	3.54±0.135***
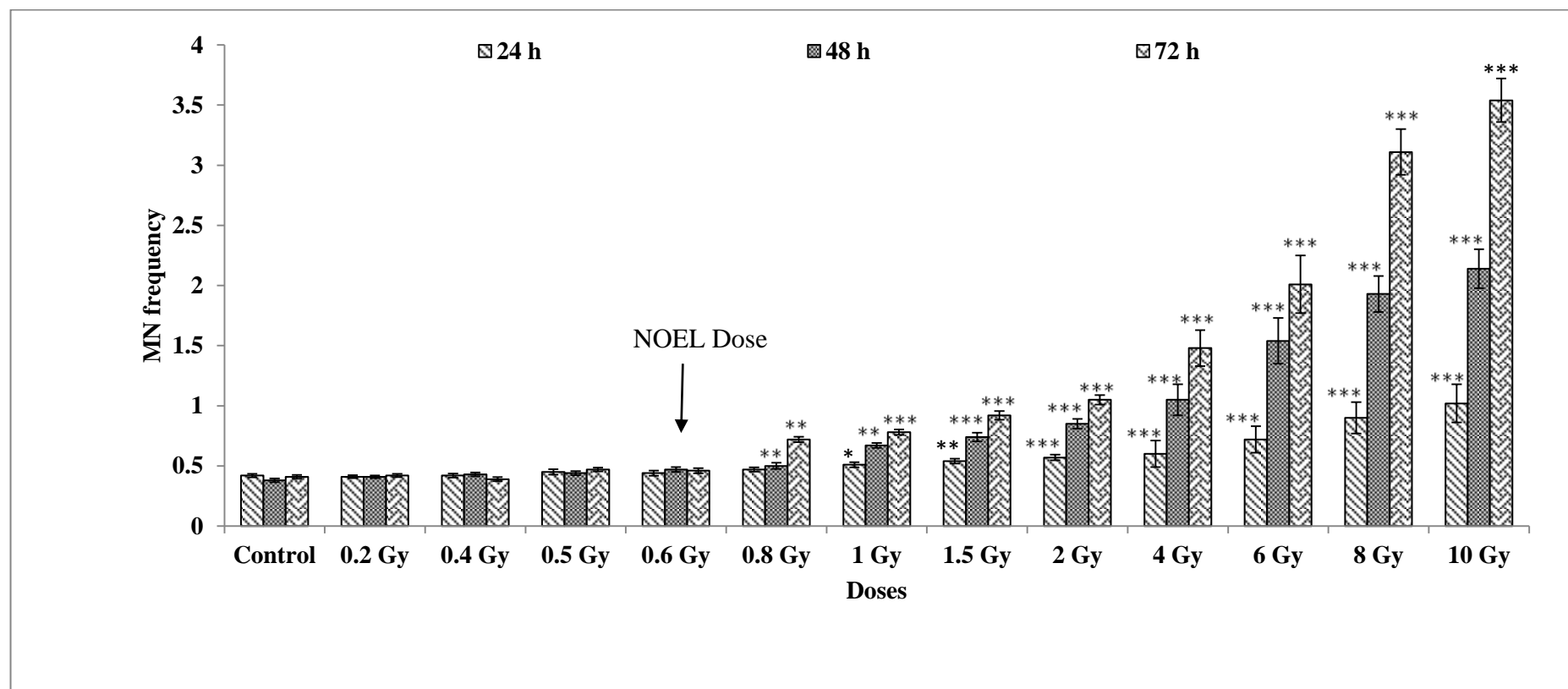
Figure.2.8: MN frequency in gill cells of zebrafish exposed to various doses of gamma radiation at 24, 48 and 72 h time intervals
Note: *p< 0.05, **p< 0.01 and ***p< 0.001

Molecular cytogenetic studies on the toxic effects of gamma radiation in Zebrafish and Common carp

2.3.3.4. Gamma radiation induced DNA damage (% tail DNA) in gill cells in zebrafish:

The DNA damage in the gill cells of gamma irradiated zebrafish at various dose (0.2, 0.4, 0.5, 0.6, 0.8 1, 1.5, 2, 4, 6, 8 and 10 Gy) in different time intervals (24 h, 48 h and 72 h) are summarized in Table 2.13 and represented graphically in Figure 2.9.

Significant DNA damage ($p<0.001$) was seen in all the fishes irradiated by various doses (0.6-10 Gy) of gamma radiation in comparison to their respective controls as per the t-test. Further, a significant dose-dependent increase (f = 526.9, $p< 0.001$) of the mean % tail DNA was observed, with a minimum (10.98 ± 0.250) at the lowest dose (0.6 Gy) and maximum (55.04 ± 6.480) at the highest dose (10 Gy) at 24h post irradiation. A similar trend was observed for the for other time intervals i.e. 48h (f = 246.2, $p< 0.001$) minimum (8.45 ± 0.361) and maximum (44.21 ± 7.450), for 72h (f = 145.7, $p< 0.001$) minimum (7.41 ± 0.280) and maximum (34.09 ± 6.570). The time-yield study indicated significant DNA damage at all-time intervals and for (0.6-10 Gy) doses studied as per the t-test. Fishes irradiated with 0.6 Gy showed the highest DNA damage (10.98 ± 0.250) at 24h post treatment, which decreased (8.45 ± 0.361) at 48h and reached the minimum (7.41 ± 0.131) by 72 h. A similar trend was observed for the other doses too i.e (0.8, 1, 1.5 2, 4, 6, 8 and 10 Gy). One way ANOVA of the DNA damage observed at different time intervals showed significant decrease in the DNA damage with the (f= = 372.9, $p< 0.001$; f= 5163, $p< 0.001$; f= 794.2, $p< 0.001$; f= 396.2, $p< 0.001$; f= 284.9, $p< 0.001$; f= 91.93, $p< 0.001$; f= 66.21, $p< 0.001$; f= 36.06, $p< 0.001$; f= 23.41, $p< 0.001$) for all doses studied (0.6, 0.8, 1, 1.5, 2, 4, 6, 8 and 10 Gy, respectively). Further, dose and time dependent was assessed by employing Two-way analysis of variance. The doses (f= 825.7, $p<$

0.001) and time (f= 514.6, *p*< 0.001) showed significant difference. The NOEL

dose of 0.5 Gy was found in which we didn't observe any significant level of

damage in gill cells of zebrafish according to micronucleus parameter.

Table. 2.13: Percentage of Tail DNA (Mean ± SD) in gill cells of zebrafish exposed to different doses of gamma radiation at different time intervals
 *p< 0.05, **p< 0.01 and ***p< 0.001

|	**24 h**	**48 h**	**72 h**
Control	7.24±0.112	7.23±0.120	7.12±0.130
0.2 Gy	7.30±0.102	7.24±0.132	7.24±0.140
0.4Gy	7.40±0.170	7.34±0.143	7.01±0.110
0.5Gy	7.42±0.142	7.38±0.131	7.25±0.131
0.6Gy	10.98±0.250**	8.45±0.361	7.41±0.131
0.8Gy	15.24±0.687**	8.12±0.593	8.02±0.415
1Gy	23.54±0.972***	12.04±0.721**	9.08±0.860*
1.5Gy	31.58±1.070***	22.65±1.610***	13.63±1.537**
2 Gy	45.02±2.960***	32.54±2.110***	19.54±1.970**
4 Gy	48.02±3.240***	35.24±2.540***	30.21±3.250***
6 Gy	49.01±3.540***	35.42±4.100***	29.54±3.980***
8 Gy	52.46±4.380***	41.02±5.410***	32.54±5.890***
10 Gy	55.04±6.480***	44.21±7.450***	34.09±6.570***
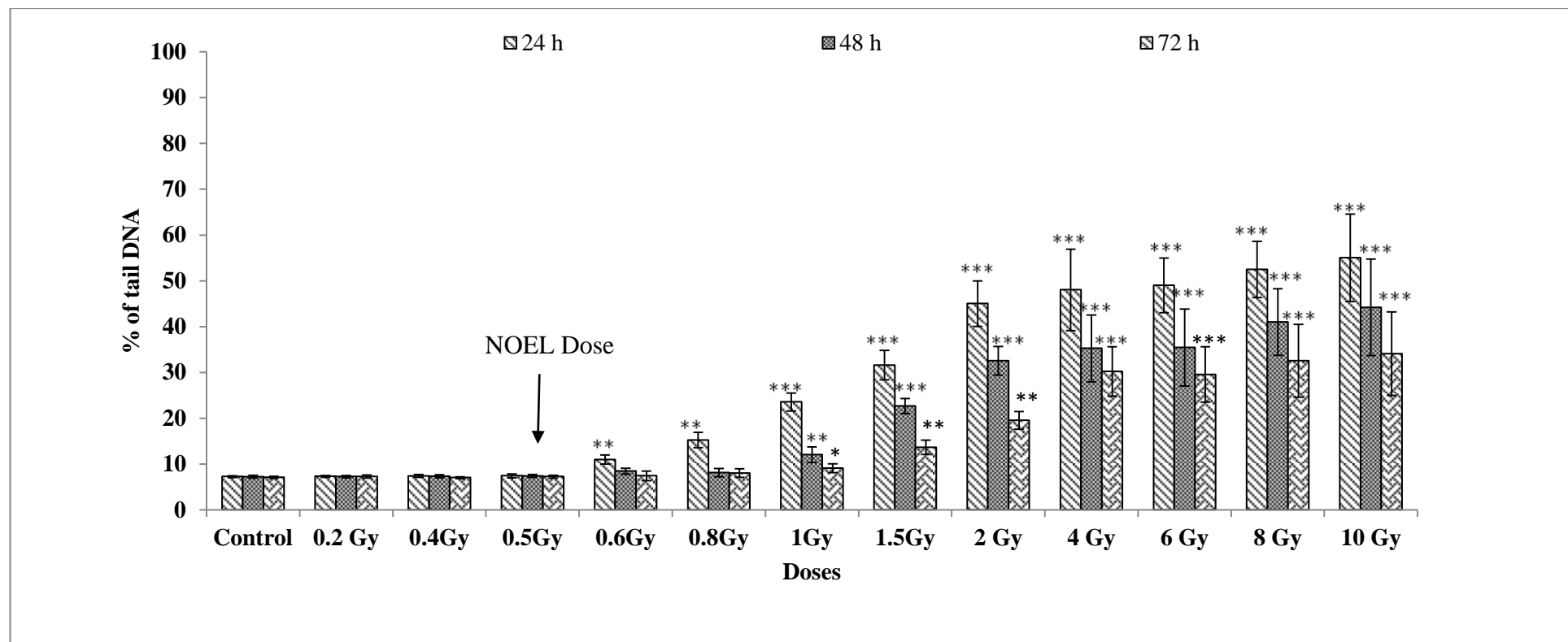
Figure.2.9: Percentage of DNA damage in gill cells of zebrafish exposed to various doses of gamma radiation at 24, 48 and 72 h time intervals
Note: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

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2.3.3.5. Gamma radiation induced DNA damage (% tail DNA) in muscle tissue of zebrafish:

The DNA damage in the muscle cells of gamma irradiated zebrafish at various dose (0.2, 0.4, 0.5, 0.6, 0.8 1, 1.5, 2, 4, 6, 8 and 10 Gy) in different time intervals (24 h, 48 h and 72 h) are summarized in Table 2.14 and represented graphically in Figure 2.10.

Significant DNA damage was seen in all the fishes irradiated by various doses (0.8-10 Gy) of gamma radiation in comparison to their respective controls as per the t-test. Further, a significant dose-dependent increase (f = 357.7, $p<$ 0.001) of the mean % tail DNA was observed, with a minimum (15.08 ± 0.870) at the lowest dose (0.5 Gy) and maximum (60.47 ± 7.585) at the highest dose (10 Gy) at 24h post irradiation. A similar trend was observed for the for other time intervals i.e. 48h (f = 649.7, $p<$ 0.001) minimum (8.04 ± 0.450) and maximum (40.25 ± 0.691), for 72h (f = 126.2, $p<$ 0.001) minimum (7.09 ± 0.461) and maximum (30.58 ± 5.452). The time dependent also indicated significant DNA damage at all-time intervals and for (0.5-10 Gy) doses studied as per the t-test. Fishes irradiated with 0.8 Gy showed the highest DNA damage ((15.08 ± 0.870) at 24h post treatment, which decreased (8.04 ± 0.450) at 48h and reached the minimum (7.09 ± 0.461) by 72 h. A similar trend was observed for the other doses too i.e (1, 1.5 2, 4, 6, 8 and 10 Gy). One way ANOVA of the DNA damage observed at different time intervals showed significant decrease in the DNA damage with the (f= 487.7, $p<$ 0.001; f= 633.1, $p<$ 0.001; f= 181.5, $p<$ 0.001; f= 205.5, $p<$ 0.001; f= 134.7, $p<$ 0.001; f= 482.8, $p<$ 0.001; f= 52.42, $p<$ 0.001; f= 79.55) for all doses studied (0.8, 1, 1.5, 2, 4, 6, 8 and 10 Gy, respectively). Further, dose and time dependent was assessed by employing Two-way analysis of variance. The doses (f= 895.6, $p<$ 0.001) and time (f= 623.2, $p<$ 0.001)

showed significant difference. The NOEL dose of 0.6 Gy was found in which we

didn't observe any significant level of damage in muscle tissue of zebrafish

according to comet asay parameter.

Table 2.14: Percentage of Tail DNA (Mean ± SD) in muscle tissue of zebrafish
exposed to different doses of gamma radiation at different time intervals
*p< 0.05, **p< 0.01 and ***p< 0.001

	24 h	48 h	72 h
Control	8.02±0.115	7.08±0.153	7.95±0.122
0.2 Gy	7.74±0.140	8.05±0.131	7.24±0.121
0.4Gy	8.08±0.213	8.04±0.230	7.98±0.153
0.5Gy	8.12±0.182	7.84±0.163	7.43±0.191
0.6Gy	8.54±3.546	7.98±2.481	7.05±1.450
0.8Gy	15.08±0.870*	8.04±0.450	7.09±0.461
1Gy	19.54±0.743**	10.19±0.605*	9.29±0.780*
1.5Gy	23.54±1.548***	15.48±1.470*	10.28±1.680*
2 Gy	30.14±2.140***	25.64±1.582***	15.24±1.201*
4 Gy	43.57±3.547***	36.54±2.580***	22.63±2.460***
6 Gy	48.21±2.680***	39.58±0.356***	26.54±0.293***
8 Gy	55.69±6.544***	40.28±4.810***	28.19±6.540***
10 Gy	60.47±7.585***	40.25±0.691***	30.58±5.452***
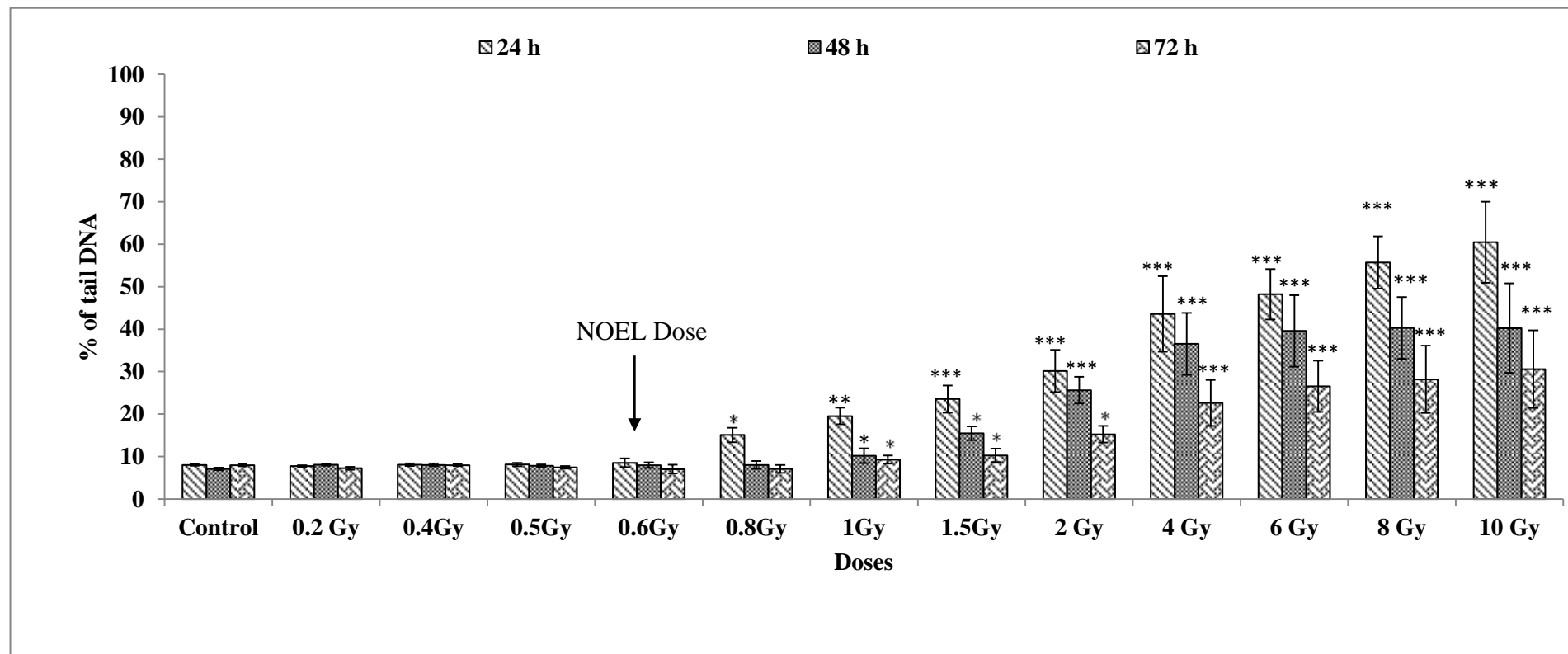
Figure 2.10: Percentage of DNA damage in muscle tissue of zebrafish to various doses of gamma radiation at 24, 48 and 72 h time intervals.

Note: *p< 0.05, **p< 0.01 and ***p< 0.001

2.3.4. Influence of different variables on DNA damage parameters (MN) in zebrafish exposed to gamma radiation:

Significance variation between tissues, time and doses of gamma radiation on DNA damage parameter (MN) in zebrafish was observed as per Multivariate analysis of variance (MANOVA) it is summarized in Table 2.15. Significant interaction between different variables in % tail DNA (Tissue, Time and Dose F = 113.65, p<0.001). The interaction of time and dose (F = 443.23, p<0.001), tissue and time (F = 1729.02, p<0.013) and tissue and dose (F = 780.53, p<0.001).

Table 2.15: Multivariate analysis of variance (MANOVA) testing the influence of fish, tissue, time and dose on MN in the fish exposed to gamma radiation

Source	Type III Sum of Squares	df	Mean Square	F	Significance
Time	367.342	2	183.671	6740.428	0.001
Treatment	1516.552	12	126.379	4637.918	0.001
Tissue	9.447	1	9.447	346.675	0.001
Time * Treatment	289.863	24	12.078	443.230	0.001
Time * Tissue	94.229	2	47.114	1729.020	0.001
Treatment * Tissue	255.227	12	21.269	780.534	0.001
Time * Treatment * Tissue	74.330	24	3.097	113.658	0.001

2.3.4. Influence of different variables on DNA damage parameters (% tail DNA) in zebrafish exposed to gamma radiation:

DNA damage parameters (% tail DNA) showed significance variation between tissues, time and doses in zebrafish as per Multivariate analysis of variance (MANOVA) it is summarized in Table 2.16. Significant interaction between different variables in % tail DNA (Tissue, Time and Dose F = 2.67, p<0.001). The interaction of time and dose (F = 48.22, p<0.001), tissue and time (F = 9.22, p<0.001) and tissue and dose (F = 38.71, p<0.001).

Table 2.16: Multivariate analysis of variance (MANOVA) testing the influence of fish, tissue, time and dose on % tail DNA in the fish exposed to gamma radiation

Source	Type III Sum of Squares	df	Mean Square	F	Significance
Time	2783.420	2	1391.710	987.026	0.001
Dose	42376.737	12	3531.395	2504.530	0.001
Tissue	62.841	2	31.420	22.284	0.001
Time * Dose	1631.896	24	67.996	48.224	0.001
Time * Tissue	52.019	4	13.005	9.223	0.001
Dose * Tissue	1310.023	24	54.584	38.712	0.001
Time * Dose * Tissue	181.027	48	3.771	2.675	0.001

2.3.4. Influence of different variables on DNA damage parameters (MN and % tail DNA) in zebrafish and common carp exposed to gamma radiation:

Multivariate analysis of variance (MANOVA) was used testing the influence of fish, tissue, time and dose on MN and % tail DNA in the both fish exposed to gamma radiation. We observed significant interaction between variables (fish, tissue, time and doses) with the dependent variable (MN and % tail DNA) is represented in the MANOVA (Table 2.17 and Table 2.18). These four variables showed significantly interaction each other on MN (F = 23.43, $p < 0.001$) and % tail DNA (F = 3.67, $p < 0.001$). Further, we also observed significant interaction between three variables in MN (Tissue, Time and Dose F = 69.26, $p < 0.001$; Fish, Time and Dose F = 30.42, $p < 0.001$; Fish, Tissue and Dose F = 86.24, $p < 0.001$; Fish, Tissue and Time F = 181.93, $p < 0.001$) and in % tail DNA (Tissue, Time and Dose F = 5.88, $p < 0.261$; Fish, Time and Dose F = 5.99, $p < 0.001$; Fish, Tissue and Dose F = 1.322, $p < 0.001$; Fish, Tissue and Time F = 64.02, $p < 0.001$). The effects of the interaction of tissue and time (F = 924.65, $p < 0.001$), tissue and dose (F = 608.86, $p < 0.001$), time and dose (F = 303.15, $p < 0.001$), fish and tissue (F = 869.41, $p < 0.001$), fish and time (F = 427.18, $p < 0.001$) as well as fish and dose (F = 19.14, $p < 0.001$) on MN. The effects of the interaction of tissue and time (F = 85.51, $p < 0.001$), tissue and dose (F = 9.73, $p < 0.001$), time and dose (F = 84.63, $p < 0.001$), fish and tissue (F = 1024.15, $p < 0.001$), fish and dose (F = 84.63, $p < 0.001$) as well as fish and time (F = 264.59, $p < 0.001$) on % tail DNA. The both parameters also showed good interaction between both common carp and zebrafish (MN: $r^2 = 0.997$; % tail DNA: $r^2 = 0.995$).

Table 2.17: Multivariate analysis of variance (MANOVA) testing the influence of fish, tissue, time and dose on MN in the fish exposed to gamma radiation

Source	Type III Sum of Squares	Df	Mean Square	F Value	Significance
Fish	1.984	1	1.984	40.013	0.001
Tissue	119.064	1	119.064	2401.798	0.001
Time	430.842	2	215.421	4345.555	0.001
Dose	3004.428	12	250.369	5050.535	0.001
Fish * Tissue	43.099	1	43.099	869.415	0.001
Fish * Time	42.354	2	21.177	427.188	0.001
Fish * Dose	11.391	12	.949	19.149	0.001
Tissue * Time	91.675	2	45.838	924.652	0.001
Tissue * Dose	362.198	12	30.183	608.866	0.001
Time * Dose	360.672	24	15.028	303.150	0.001
Fish * Tissue * Time	18.038	2	9.019	181.938	0.001
Fish * Tissue * Dose	51.302	12	4.275	86.240	0.001
Fish * Time * Dose	36.200	24	1.508	30.427	0.001
Tissue * Time * Dose	82.408	24	3.434	69.265	0.001
Fish * Tissue * Time * Dose	27.882	24	1.162	23.435	0.001

Note: R Squared = 0.997

Table 2.18: Multivariate analysis of variance (MANOVA) testing the influence of fish, tissue, time and dose on % tail DNA in the fish exposed to gamma radiation

Source	Type III Sum of Squares	Df	Mean Square	F value	Significance
Fish	7116.492	1	7116.492	3846.693	0.001
Tissue	4033.558	2	2016.779	1090.134	0.001
Dose	8226.269	2	4113.134	2223.282	0.001
Time	5421.21	12	9768.981	5280.449	0.001
Fish * Tissue	3789.418	2	1894.709	1024.151	0.001
Fish * Dose	310.460	2	155.230	83.907	0.001
Fish * Time	5874.147	12	489.512	264.597	0.001
Tissue * Dose	72.024	4	18.006	9.733	0.001
Tissue * Time	3796.818	24	158.201	85.513	0.001
Dose * Time	3757.783	24	156.574	84.633	0.001
Fish * Tissue * Dose	9.785	4	2.446	1.322	0.261
Fish * Tissue * Time	2842.757	24	118.448	64.025	0.001
Fish * Dose * Time	266.200	24	11.092	5.995	0.001
Tissue * Dose * Time	522.415	48	10.884	5.883	0.001
Fish * Tissue * Dose * Time	326.321	48	6.798	3.675	0.001

Note: R Squared = 0.995

2.3.5. NOEL (No Observed Effect Level) dose estimation:

In the present study NOEL (No Observed Effect Level) dose was ascertained for various tissues of both the species of fishes. NOEL dose varied from tissue to tissue, species to species as well as from one parameter to the other. The lowest doses at which effects have been observed. Thus, although both fishes belong to the same family (Cyprinidae) they showed some different sensitivity to radiation at the tissue specific level.

2.4. Discussion:

Fishes represent an excellent model organism for studying the toxic and mutagenic potential of pollutants in water samples (Belpaeme et al. 1996). The significant increase of micronuclei and DNA damage encountered in gamma irradiated fishes in the present study indicate the mutagenic / genotoxic potential of gamma radiation in the freshwater fish common carp and zebrafish*,* as well as, the possible use of common carp and zebrafish for the assessment of status of pollution of freshwater bodies by radioactive materials.

2.4.1. LD$_{50/30}$ value:

The LD$_{50/30}$ study was carried out for both the carp species of fish and it was found to be 21.6 Gy for common carp and 20.4 Gy for zebrafish in the present study. This value is very close to the LD$_{50/30}$ value of gamma radiation exposed fish *Catla catla* (22.38 Gy) (Anbumani and Mohankumar (2012) and gold fish exposed to ionizing radiation (23 Gy) (Driver, 1994). These findings may indicate that closely related members of the same family Cyprinidae of fishes may exhibit almost similar LD$_{50}$ values, which in turn may be due to

their biochemical / physiological / metabolic similarities and phylogenetic relationship.

2.4.2. Gamma radiation induced micronuclei:

The significant increase in the frequency of MN observed at all the doses of gamma radiation in the present study indicates the mutagenic potential of gamma radiation in common carp and zebrafish. Anbumani and Mohankumar (2011, 2012) reported a similar observation in a closely related fish *Catla catla* which were also exposed to gamma radiation. This may indicate almost similar kind of genotoxic effect of gamma radiation in common carp, zebrafish and *Catla catla*, which in turn may be due to their phylogenetic relationship as they belong to the family Cyprinidae. Increased micronuclei may be due to DNA double-strand breaks induced by gamma radiation which lead to symmetrical / asymmetrical chromatid and chromosome exchanges or fragments that failed to be included in the daughter nuclei at the completion of telophase during mitosis due to lack of spindle attachment during the segregation process in anaphase (Fenech, 2011).

The dose-dependent increase in the frequency of MN observed in the erythrocytes and gill cells of gamma irradiated common carp and zebrafish in the present is in agreement with the similar findings of Gustavino et al. (2001) in the erythrocytes of *C. carpio* exposed to various doses of X-rays. These may indicate a similar mode of action of the both ionizing radiations viz. X-ray and gamma rays on the DNA of common carp. Our study is also supported by the *in situ* observations of Sugg et al. (1996) and Ilyinskikh et al. (1998) wherein they found a significant increase of MN in the fishes *Ictalurus punctatus* and *Esox lucius* respectfully collected from nuclear contaminated sites. *In vitro* study by Cassidy et al. (2007) in the fish cell lines exposed to

low-dose gamma radiation also indicated a dose-dependent increase of MN. These dose dependent increases in the frequency of MN may be due to the induction of more double strand DNA breaks by higher doses of gamma radiation, their failure to get repaired and the production of micronuclei.

The time-dependent increase of MN observed in the erythrocytes and gill cells of gamma irradiated common carp and zebrafish in the present study is on par with the findings of Anbumani and Mohankumar (2012) in *Catla catla* exposed to gamma radiation and *C. carpio* exposed to X-rays (Gustavino et al., 2001), which may indicate the mutagenic potential of ionizing radiation in Cyprinids. Inhibition of apoptosis is one of the causes which supplement increased frequency of MN as demonstrated by Decordier et al. (2002) and confirmed by Polard et al. (2011). Further, it may also be due to the continued induction of double strand breaks by the bystander effect of acute exposure to gamma radiation and the failure of their repair resulted in the increased frequency of MN in the present study.

2.4.3. Gamma radiation induced DNA damage:

Significant increase of % tail DNA damage observed in the present study indicates the genotoxic potential of gamma radiation in freshwater fish common carp and zebrafish. Lemos et al. (2017) also observed significant level of DNA damage in the blood cells of zebrafish, which were exposed to various doses of ionizing radiation. Further, increased DNA damage was also reported by Jarvis and Knowles (2003) in zebrafish larvae exposed gamma radiation. Anbumani et al. (2012) and Anbumani and Mohankumar (2015) observed increased DNA damage in *Catla catla* exposed to gamma radiation. Our present findings along with other above cited reports suggest that the Cyprinids in general exhibit similar mode of action against ionizing radiation.

The dose dependent increase of DNA damage induced by gamma radiation in the present study is in agreement with the findings of Lemos et al. (2017) as well as that of Jarvis and Knowles (2003) in zebrafishes exposed *in vivo* to various doses of ionizing radiation. Further, *in vitro* study of gamma irradiated zebrafish embryonic cells (ZF4) also showed increased DNA damage (Sandrine et al., 2011). All these findings predict almost similar level of DNA damage induced by gamma radiation both *in vivo* and in *vitr*o, as well as, in larvae and adult zebra fish.

The time dependent decrease in DNA damage observed at a later time in the present study in alkaline comet assay is in agreement with the findings of Lemos et al. (2017). Further, Anbumani et al. (2012) and Anbumani and Mohankumar (2015) where in they observed a time dependent decrease of DNA damage in fish *Catla catla* exposed to gamma radiation using flowcytometry. Similar reduction of DNA damage at later time intervals was also observed by Praveen Kumar et al. (2014) in which bivalves were exposed to various doses of gamma radiation. Time dependent decrease in DNA damage were also reported by several scientists using chemical mutagens (Rank and Jensen, 2003; Sharma et al., 2007; Augustyniaka et al., 2015). This time dependent decrease of tail DNA may indicate that the single strand damage induced by gamma radiation gets repaired by the inherent cellular DNA repair mechanisms. However, multiple reasons are cited for this by various scientists. Decrease in genetic damage at later time intervals may be due to the progress of DNA repair processes or elimination of the cells / damaged genetic materials by any of the processes such as apoptosis / degradation/degeneration (Shyama and Rahiman, 1993; Banu et al., 2001; Preeti and Shyama, 2009; Anbumani et al., 2012).

The gill is considered to be the prime target for toxicants because of its large surface area and additionally its repair system is not as effective as that of other tissues (Pandey et al., 2008; Li et al., 2009; Oliveira et al., 2009; Li et al., 2010). UV-irraditation also severely damages the gill epithelium and may lead to dysfunctions in respiration and osmoregulation in affected fish (Sharma and Chakrabarti, 2006). The DNA damage by gamma radiation in gill cells may affect the respiratory function and may also affect one of the most important physiological processes i.e. osmoregulation. O'Dowd et al. (2006) reported that rainbow trout gill tissues produced significant bystander effects after irradiation and it may be one of the reasons of higher DNA damage observed in the present investigation. Severe histological changes were induced by gamma radiation in aquatic organisms (Sadiq et al., 2012; Stalin et al., 2013a, 2013b). As a consequence, the DNA damage in the gills may directly or indirectly be affected due to these histological changes. Muscles are two principal types, viz. i. fast twitch or white muscle, and ii. slow-twitch or red muscle. Undulatory swimming in fish is powered by the segmental body musculature of the myotomes. DNA damage in the muscle can impact on functioning of muscle tissue in fish and can thus lead to abnormalities in swimming patterns (Altringham and Ellerby, 1999). The observed tissue specific response may be due to physiochemical activities distinctive to these organs, with respect to either the activation or detoxification of pollutants or the repair of different types of strand breaks.

The mutagenic effects of radiation being of a stochastic nature, it is to be expected that the induced incidence would be linear with low acute doses. However, reports on the lowest doses of radiation at which toxic effects are

not observed in fishes are lacking. In contrast, the present study we found NOEL dose for common carp and zebrafish in respect to particular parameter. We observed NOEL dose varied from tissue to tissue, species to species as well as from one parameter to the other. DNA damage is significantly influenced by different doses, time intervals, different tissues as well as fish species in the present study as observed by the MANOVA which shows that both the fishes respond in a similar manner to the effects of gamma radiation. Additionally, it is seen that genotoxicity is not only influenced singly by time, dose and tissue type, but also by the various interactions of these parameters. Thus the effects of gamma radiation may be manifested in each fish by interactions between dose and time, dose and tissue type, time and tissue type or an overall interaction of dose, time and tissue type. Both the fishes can be used as model organisms to monitor radiation pollution in the environment. Further, tissue-specific responses can also be used to assess the sensitivity of different tissues to gamma radiation. The micronucleus test and comet assay therefore represent essential biomonitoring tools to evaluate gamma radiation exposure and pollution in the environment.

2.5. Summary:

The present study was undertaken to evaluate the gamma radiation-induced DNA damage in the freshwater fish common carp and zebrafish and thereby to findout the NOEL. The $LD_{50/30}$ study was carried out for both the above species of fish and it was found to be 21.6 Gy for common carp and 20.4 Gy for zebrafish. Fishes were irradiated with doses (0.2, 0.4, 0.5, 0.6, 0.8, 1, 2, 4, 6, 8 and 10 Gy) of gamma radiation and their genotoxic effects in different tissues (blood, gill and muscle) were studied employing micronucleus (MN) and comet assays (Alkaline single gel electrophoresis). A

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significant number of MN were observed in both, the dose and the time dependent studies. The lowest frequency of MN was observed at 24h post treatment, which increased considerably at the later time intervals and reached the maximum by 72 h. The result may indicate that the double-stranded DNA damage might not have been repaired, as indicated by increased micronuclei at later periods. A significant increase in DNA damage was observed as indicated by the increase of % tail DNA damage at all the doses of gamma radiation as compared to controls in both fish species. This showed a dose-dependent increase of genetic damage induced in fishes by gamma radiation. Further, the highest DNA damage was observed at 24 h which gradually decreased with advancement of time i.e. at 48 and 72 h in both species of fishes. This may indicate repair of the damaged DNA and/or loss of heavily damaged cells as the post irradiation time advanced. The present study reveals that gamma radiation induces single strand breaks in DNA as measured by alkaline comet assay.

Further, the NOEL (No Observed Effect Level) dose was ascertained for various tissues of both the species of fishes. NOEL dose varied from tissue to tissue, species to species as well as from one parameter to the other. In the micronucleus test, NOEL dose observed for erythrocytes and gill cells of common carp were 0.6 Gy and 0.4 Gy, respectively and that of zebrafish was 0.6 Gy for both the types of tissues. In the comet assay, NOEL dose observed in common carp erythrocytes, gill cells and muscle cells were 0.5 Gy, 0.4 Gy and 0.4 Gy respectively whereas it was 0.5 Gy for zebrafish erythrocytes and gill cells and 0.6 Gy for muscle cells. Thus, although both fishes belong to the same family (*Cyprinidae*) they showed different sensitivity to radiation at the

tissue specific level. Significant interaction between different dose, time intervals and different tissue in both fishes in the present study shows that both the fishes respond in a similar manner to the effects of gamma radiation. Both the fishes can use as model organisms to monitor radiation pollution in the environment. The micronucleus test and comet assay therefore represent essential biomonitoring tools to evaluate gamma radiation exposure and pollution in the environment.

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3.1. Introduction:

Aquatic organisms often get exposed to several hazards, which may be chemical, physical or biological contaminants in their aquatic environment. The introduction of chemicals into the environment by anthropogenic activities often represents a serious risk to environmental and human health. Zebrafish (*Danio rerio*) is gaining importance as a popular model organism and is widely employed in developmental biology, physiology, toxicology, environmental research, cancer research and ecotoxicological studies, especially for the biomonitoring of pollution in water bodies. Developmental toxicology is a subdivision of reproductive toxicology and is defined as the study of the adverse effects on the developing embryo of an organism that results from exposure prior to conception (either parent) as well as during the prenatal or postnatal development. Developmental toxicity studies are normally designed to access development *in utero*. The major manifestations of developmental toxicity include death and development of the organism, structural abnormalities, altered growth and functional deficiencies. Morphological abnormalities routinely studied in fish include colour change, weight loss, exopthalmia, distended stomach, hemorrhage / redness, gas bubbles, fin erosion or lesions and skin ulceration. Survival studies routinely carried out on the adult fish include fecundity, which is expressed on the basis of the number of eggs laid by surviving females per reproductive test and fertilization success, which is determined by whether the egg will undergo late cleavage and determine whether the fertility rate is easily achieved. Precise biomonitoring methods and biomarkers are the need of the day to assess the impact of radiation exposure high/low levels in the aquatic environment. Zebrafish embryos are ideal for evaluating genotoxic stress as well as radiation-related studies (Geiger et al., 2006; Choi et al., 2012a, b, 2013a, b; Marone et al.,

Molecular cytogenetic studies on the toxic effects of gamma radiation in Zebrafish and Common carp

2014; Pereira et al., 2014; Choi and Yu., 2015). FASSET has strongly recommended the need to undertake more systematic studies on the effect of radiation on fish eggs as a separate reference organism (FASSET Project, 2001).

3.1.1. The zebrafish embryo model:

Zebrafish (*Danio rerio*) is used as a model system to detect the presence of toxic agents in water, involving studies in toxicology, mutagenicity, development and carcinogenicity (Hill et al., 2005; Lawrence, 2007; Bourrachot et al., 2008; 2009; Zhou et al., 2014, 2015). It is used extensively as a vertebrate model for toxicology studies because of their short breeding cycle, fast rate of development, lower husbandry cost, small size and completely sequenced genome (Samson and Shenker, 2000; Hallare et al., 2006; Fako et al., 2009; Lin et al., 2013). Zebrafish eggs contain two distinct membranes, i.e. the outer chorionic membrane and the inner vitelline membrane. In between two membranes there is a perivitelline space filled with a viscous fluid. The chorion is the first barrier, on which numerous pore canals are distributed (Rawson et al. 2000; Lee et al. 2007). Zebrafish embryos are transparent throughout their various developmental stages, which facilitate direct optical observation of the toxic effects on their internal organs without disturbing the embryos. The embryonic development is almost similar to the embryogenesis of higher vertebrates including humans and occurs rapidly. Kimmel et al. (1995) have described seven major developmental stages of the embryo of zebrafish [zygote, cleavage, blastula, gastrula, segmentation (10 and 24 hours of post fertilization (hpf), pharyngula and hatching period (42 and 72 hpf)] that occur during the first 3 days after fertilization. Many of the complex variety of morphogenetic movements, the rudiments of the primary organs, the development of somites, the tail bud and the elongation of embryo were observe at 24 hpf. The first stages of

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development are completed in the first 24 hpf, the normal embryo hatches by 48-72 hpf and fully develops at 120 hpf (Hoar and Randall, 1988; Kunz, 2004; Gong and Korzh, 2004; Rubinstein, 2006). The transparent embryos develop *ex utero*, facilitating visual recording of organogenesis (Asharani et al., 2009). The cells in the developing embryos especially at early stages are sensitive to genotoxicants and chemicals, as they have no active immune system to fight the foreign bodies (Ikegami et al., 1997; Bai et al., 2010). Additionally, any chemical that damages DNA in the early developmental stages might induce phenotypic defects due to the absence of cell cycle checkpoints and active DNA repair pathways (Ikegami et al., 1997; Asharani et al., 2009).

Further, zebrafish can be used as a test organism to assess the effects of environmental pollution on both aquatic and terrestrial vertebrates due to similarities in their development and cellular composition of major organ systems (Cristina et al., 2009). The organ physiology of zebrafish and other vertebrates is also similar and has a high degree of genomic homology to humans thereby, thus making it a good model for identifying potential health effects in humans. Chemically induced malformations in the brain, jaw, eye, heart, yolk sac, notochord, trunk and tail are directly observable under a dissection microscope (Kim et al., 2013). Additionally, fishes are renowned for their ability to bio-accumulate trace contaminants in the environment and eventually passing them to organisms at higher trophic level in the food web. Therefore, the zebrafish embryo has been proven to be a good model vertebrate to assess the toxicity of contaminants/pollution (Cheng et al., 2007; Lee et al., 2007; Usenko et al., 2007; Zhu et al., 2008). The major advantage of the use of early life stages of zebrafish for toxicological studies is that it will save a lot of time when compared to the use of other higher models such as mice, for these studies.

3.1.2. End points for toxicity studies:

An extensive evaluation of the potential effects of toxicants on organisms requires investigation of impacts at different levels of biological organization (Mothersill and Seymour, 2012). Developmental toxicity is a very sensitive endpoint for measuring the toxic effects of radiation (IAEA, 1992). Many scientists recommended to assessing the toxic effects of contaminants in the early life stages (ELS) of fish as an experimental model (McAleer et al., 2005; Fraysse et al., 2006; Bourrachot et al., 2008). Mortality, morbidity and reproduction (fecundity and fertility rates) of individuals are the major endpoints often used To evaluate toxic effects of radiation morbidity mortality, and reproduction (fecundity and fertility rates) of individuals are used as the major endpoints (Woodhead, 2003). Investigations into the biological effects of radionuclides are focused on 4 endpoints: mortality, morbidity, reproduction and mutation (Larsson, 2008). More experimental data on these endpoints are required in most groups of organisms (non-human biota), including fish (Woodhead, 2003; Real et al., 2004; Copplestone et al., 2008; Dallas et al., 2012).

Gamma irradiation is known to induce repairable as well as non-repairable lesions in the DNA and its lethal effect will depend on the competing processes of repair and mis-repair of DNA (Minouflet et al., 2005). International Commission on Radiological Protection (ICRP, 2002) strongly recommended the need to include studies on molecular effects, such as DNA damage, as endpoints for toxicity studies. Comet assay is as a simple and rapid method to evaluate the DNA damage (Jarvis and Knowles, 2003; Riganoto et al., 2005; Kosmehl et al., 2006; Praveen Kumar et al., 2014).

3.1.3. Toxic effects of radiation on embryos:

The effects of radiation on biological systems have been studied for many years and it is now accepted that direct damage to DNA from radiation is the triggering event leading to various biological effects. Embryos and larvae have been often employed in toxicity studies of physical/chemical agents due to their sensitivity and visible developmental abnormalities. The nature and extent of the toxic effects mainly depend on the developmental stage, the early embryonic stages being more vulnerable to ionizing damage (Walker and Streisinger, 1983; Haggeret al., 2005; McAleer et al., 2005), including mutations, apoptosis and oxidative stress responses (Yabu et al., 2001).

Dethlefsen et al. (2001) observed loss of buoyancy, increased mortality and reduced viable hatching as the effect of UV radiation on eggs of dab (*Limanda limanda*) and plaice (*Pleuronectes platessa*). Torres et al. (2012) studied the implications of the molecular response to UV radiation exposure in fish embryos and observed reduced survival rate and occurrence of developmental abnormalities (mainly caudal posterior notochord bending/torsion). Sayed and Mitani, (2016) observed destructive effects of ultraviolet A on *Oryzias latipes* such as decreased hatching rate, increased mortality rate and increased morphological deformities. Ultraviolet A induced morphological and histological malformations during embryogenesis of *Clarias gariepinus* (Burchell, 1822) was observed by Mahmoud et al. (2009). X-rays irradiated male medaka (*Oryzias latipes*) showed heritable malformations in the progeny (Ishikawa and Hyodo-Taguchi, 1997). Miyachi et al. (2003) studied the effect of X-rays on the cleavage stage of zebrafish embryo and found a significant delay in hatching. Induction of DNA damage by ionizing radiation has been reported in zebrafish embryos, as an *in vivo* model (Bladen et al., 2005; McAleer

et al., 2005, 2006; Daroczi et al., 2006; Geiger et al., 2006). Yum et al. (2007) observed the hermetic effect of low-doses of alpha particles on dechorionated zebrafish embryos at 1.5 h post fertilization (hpf). Multiple developmental abnormalities were induced by acute irradiation in medaka (*O. latipes*) embryos (Yasuda et al., 2006; Kuhne et al., 2009), whereas a few morphological abnormalities were observed in chronic irradiated embryos (Hyodo-Taguchi and Etoh, 1993). The majority of the available reports on the effect of gamma radiation on zebrafish embryo and zebrafish cell lines are mainly based on chronic exposures (Ryan et al., 2008; Pereira et al., 2011; Simon et al., 2011).

3.2. Materials and methods:

The main objective of the present study was to assess the toxic effects of acute gamma radiation on zebrafish (*Danio rerio*) embryo using various developmental toxicity endpoints viz. mortality rate, hatching rate, morphological malformations, body length, DNA single strand breaks.

3.2.1. Fish maintenance and egg production:

Juvenile zebrafish *(Danio rerio)* (males and females) were procured from Aquaculture farm, Margao, Goa, sexed and maintained separately as stock in aquaria fitted with aerators and heaters at 28±1ºC with 14:10 (light: dark) photoperiod and acclimatized at least for a week (Plate 3.2). Water was manually renewed by replacing 50% of the total volume once in every week with fresh water and also by refilling the evaporated water every day. Fishes were fed twice daily with live brine shrimps (*Artemia salina*) and commercial fish feed (Brand et al., 2002). As and when the eggs were needed for studies, adult male and female fish (ratio 1:2) were placed in a hatching box in the late evening and allowed to remain together overnight for spawning. Spawning process was triggered in the early morning hours of the day by putting on the lights which lasted around one

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hour. Viable eggs were collected and rinsed at least three times with E3 medium. E3 medium was prepared by dissolving various salts in the standard hatchery water as per Brand et al. (2002) (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$) with pH 7.2–7.3, dissolved oxygen 6.3 mg/L, total hardness 65 mg/L (as $CaCO_3$) and temperature 28 ± 1ºC. All the chemicals used were of analytical grade. In order to ensure developmental synchronization at the beginning of exposure, the embryos at the blastula stage of about 3 hours post fertilization (hpf) were sorted under a stereo-microscope and employed for irradiation (Plate 3.3)

3.2.2. Irradiation:

The assay was mainly based on the embryo test procedure developed by OECD guidelines (2013). In brief, embryos (3hpf) were transferred into a 96-well multiplate, one embryo per well filled with E3 medium and irradiated. One time irradiation was done from a Co^{60} teletherapy unit at Goa Medical College, Goa for the appropriate length of time based on dose rate on the day of dosing (acute exposure) to result in doses of 0.1, 0.25, 0.5, 1, 2.5, 5, 7.5 and 10 Gy of gamma rays. Parallel control groups were mock irradiated by placing the samples in the irradiator. Both the control and irradiated embryos were maintained at 28 ± 1ºC with a 14:10 hrs (light: dark) photoperiod. E3 media were renewed regularly at 24 h intervals of time (Plate 3.4).

3.2.3. Endpoints:

Mortality, hatching rate, malformations and body length of the larvae were employed as the toxicological endpoints at the individual level for the present study. The embryos in the well were directly observed under a stereo

microscope connected to a camera and the above endpoints were scored at 24, 48, 72, 96 and 120 hpf (Plate 3.5).

The mortality rate is calculated as the number of dead embryos observed at each time interval divided by the number of embryos used for the experiment at the beginning.

$$\textbf{Mortality Rate (\%)} = \frac{\text{No. of embryo/ larvae dead}}{\text{No. of embryos used}} \times 100$$

The hatching time viz. the proportion of embryos hatched at each time period is calculated as the number hatched at each time interval divided by the number of zebrafish observed alive at that time period.

$$\textbf{Hatching time (\%)} = \frac{\text{No. of embryos hatched each time}}{\text{No. of embryos alive each time}} \times 100$$

HT_{50} was calculated as the time necessary for 50% of the eggs to hatch in each experimental condition.

The hatching rate is calculated as the total number of embryos hatched at 120 hpf divided by the number of embryos taken for the experiment.

$$\textbf{Hatching Rate (\%)} = \frac{\text{No. of embryos hatched at 120 hpf}}{\text{No. of embryos used}} \times 100$$

Larvae of 120 hpf were positioned on the lateral side, photographed and their body length was measured.

The frequency of morphological deformities in embryos was calculated as the total number of larvae with morphological deformities at 120 hpf divided by the number of zebrafish which were alive.

No. of larvae with morphological deformities

Morphological deformities (%) = --- x100
No. of larvae alive

3.2.4. Comet assay:

The genotoxic effect induced by gamma irradiation in the various early developmental stages of zebrafish at the molecular level/DNA was evaluated employing the alkaline comet assay as per Singh et al. (1988) with slight modifications. Single cell isolation was carried out as described by Kosmehl et al. (2006). Embryos which had a minimum of 90% cell viability were selected for the comet assay. Two hundred cells were scored from each of the five slides per group. Percentage tail DNA, which is considered as the most reliable parameter (Emmanouil et al., 2007) was recorded. All the experimental and control groups were represented in three replicates.

3.2.5. Statistical analysis:

Analysis of the data obtained was carried out using the statistical package GraphPad Prism 5.0 (GraphPad Software, Inc. CA 92037, USA). The control and experimental values obtained at various time intervals (24, 48, 72, 96 and 120 hpf) for hatching rates and mortality were compared pairwise using pairwise chi square test. In order to keep a global alpha risk at 5% level for each observation time, *p*-values of the pairwise Chi-square tests were adjusted according to the Holm-method (Holm, 1979). Comet assay data (% tail DNA) were arcsine transformed and tested for normality and homogeneity using the Shapiro-Wilk test and Levene's test respectively. Data of genotoxicity endpoints (comet assay), morphological deformities, body length, hatching rate and median hatching rate were analyzed by one-way ANOVA, with a post hoc pairwise Tukey test to identify differences between specific treatment groups.

3.3. Results:

3.3.1. Mortality:

The mortality induced by various doses of gamma radiation (0.1-10 Gy) in zebrafish embryos at various time intervals are shown in Table 3.1. The control group of embryos (0 Gy) exhibited an overall mortality of less than 2%. A dose dependent increase of mortality was observed in irradiated zebrafish embryos. Embryos irradiated with 0.5 to 10 Gy of gamma radiation exhibited significantly increased mortality at all the time intervals of study (24 to 120 hpf) as per Chi-square test (Figure 3.1). Significant mortality was seen in zebrafish embryos irradiated with various doses of gamma radiation compared to their respective controls. Further, a significant dose-dependent increase (F = 900, $p < 0.001$) of the mortality rate was observed, with 10.07 ± 1.59, 15.28 ± 1.59, 18.40 ± 1.20, 19.79 ± 1.04, 23.26 ± 0.60, 36.81 ± 1.20, 46.18 ± 2.40 and 75 ± 1.80 % at the dose 0.1, 0.25, 0.5, 1, 2.5, 5, 7.5 and 10 Gy at 120hpf (Table 3.2; Figure 3.2).

Table 3.1: Mortality rate (%) of embryos irradiated with various doses of gamma radiation at different time intervals (Mean ± SD).

	24 hpf	**48 hpf**	**72 hpf**	**96 hpf**	**120 hpf**
Control	1.04 ± 0	1.04 ± 0	1.73 ± 0.6	1.74 ± 0.61	1.73 ± 0.6
0.1 Gy	1.73 ± 0.60	4.16 ± 1.04	5.21 ± 1.20	7.28 ± 1.591	10.07 ± 1.59
0.25Gy	4.516 ± 0.61	6.25 ± 1.04	7.99 ± 0.60	8.34 ± 1.03	15.28 ± 1.59
0.5 Gy	6.946 ± 1.58	10.07 ± 1.59	12.16 ± 1.20	14.58 ± 0.60	18.66 ± 1.20
1 Gy	10.07 ± 1.59	13.89 ± 1.20	14.59 ± 0.61	17.01 ± 1.03	19.79 ± 1.04
2.5 Gy	12.85 ± 0.60	15.28 ± 0.61	16.33 ± 0.60	18.75 ± 0.60	23.26 ± 0.60
5 Gy	15.63 ± 1.03	20.84 ± 2.08	28.83 ± 1.59	31.25 ± 1.20	36.81 ± 1.20
7.5 Gy	17.71 ± 1.04	25.70 ± 2.16	29.87 ± 1.59	45.14 ± 1.80	646.18 ± 2.40
10 Gy	24.31 ± 1.59	44.45 ± 3.18	50.01 ± 1.60	63.89 ± 2.75	75.0 ± 1.80

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Figure 3.1: Mortality rate (%) of embryos irradiated with various doses of gamma radiation at different time intervals (Mean ± SD). Significant differences between the control and irradiated groups were determined using Chi-square test. Note: @ = $p < 0.01$, $ = $p < 0.001$

Table 3.2: Mortality rate (%) of embryos irradiated with various doses of gamma radiation at 120 hpf (Mean ± SD).

	120hpf
Control	1.73 ± 0.6
0.1 Gy	10.07±1.59
0.25Gy	15.28±1.59
0.5 Gy	18.66±1.20
1 Gy	19.79±1.04
2.5 Gy	23.26±0.60
5 Gy	36.81±1.20
7.5 Gy	646.18±2.40
10 Gy	75.0±1.80
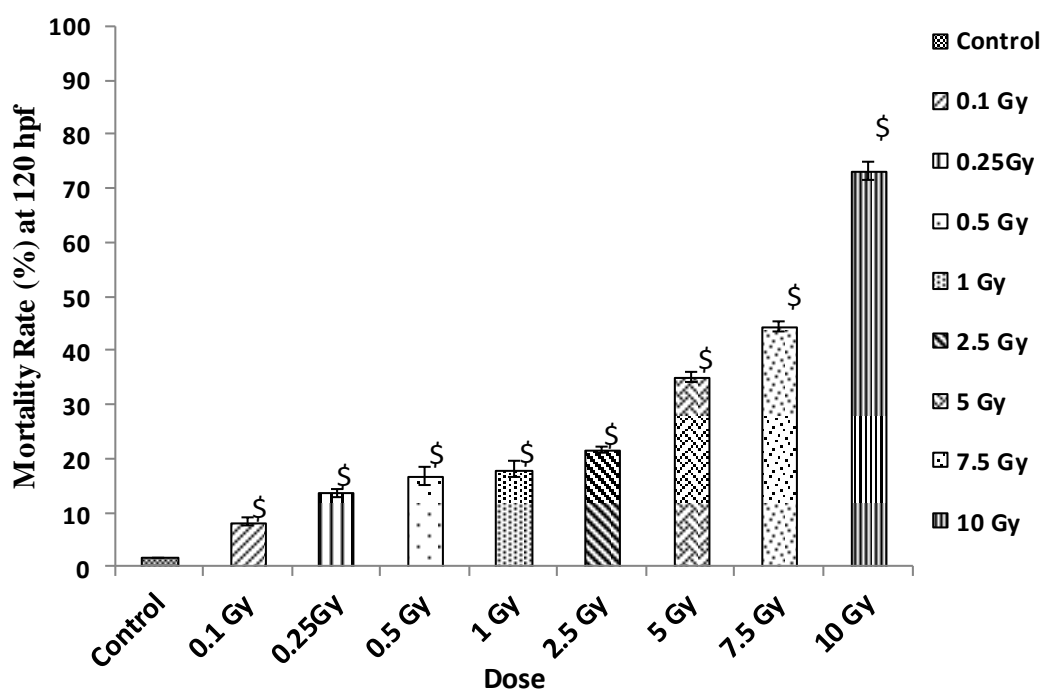
Figure 3.2: Mortality rate (%) of embryos irradiated with various doses of gamma radiation at 120 hpf (Mean ± SD). ($ P<0.001 denotes statistically significant difference from the control, Tukey's test of significance).

3.3.2. Hatching time and rate:

The proportion of eggs hatched at various time intervals are presented in Table 3.3 and graphically shown in Figure.3.3. Significant decrease in hatching rate of gamma radiation exposed embryos was noted as the observation time advanced. Further, the hatching rate decreased as the irradiation dose increased. Both the control and irradiated embryos started hatching at 48 hpf. All these control embryos hatched by 96 hpf, whereas all the survived irradiated embryos hatched only by 120 hpf. Embryos irradiated with various doses of gamma radiation showed significantly reduced hatching rate at 48 hpf as well as at 72hpf. However, they failed to show significant reduction in hatching rate at later time intervals viz. 96 and 120 hpf. The hatching process was not synchronous with the median hatching time [HT_{50} (95% CI)] is calculated and summarized in Table

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3.4. The value for control group (50.90 ± 0.55) and it increased significantly (F =167.5, $p < 0.001$) with increasing doses of gamma radiation (63.07 ± 1.25, 66.80 ± 1.57, 66.93 ± 1.16, 67.43 ± 0.80, 68.17 ± 0.77, 68.70 ± 0.55, 69.43 ± 1.15 and 82.10 ± 2.72) for 0.1, 0.25, 0.5, 1, 2.5, 5, 7.5 and 10 Gy respectively (Figure3.4).

Table 3.3: Hatching rate (%) of embryos irradiated with various doses at different time intervals (Mean ± SD).

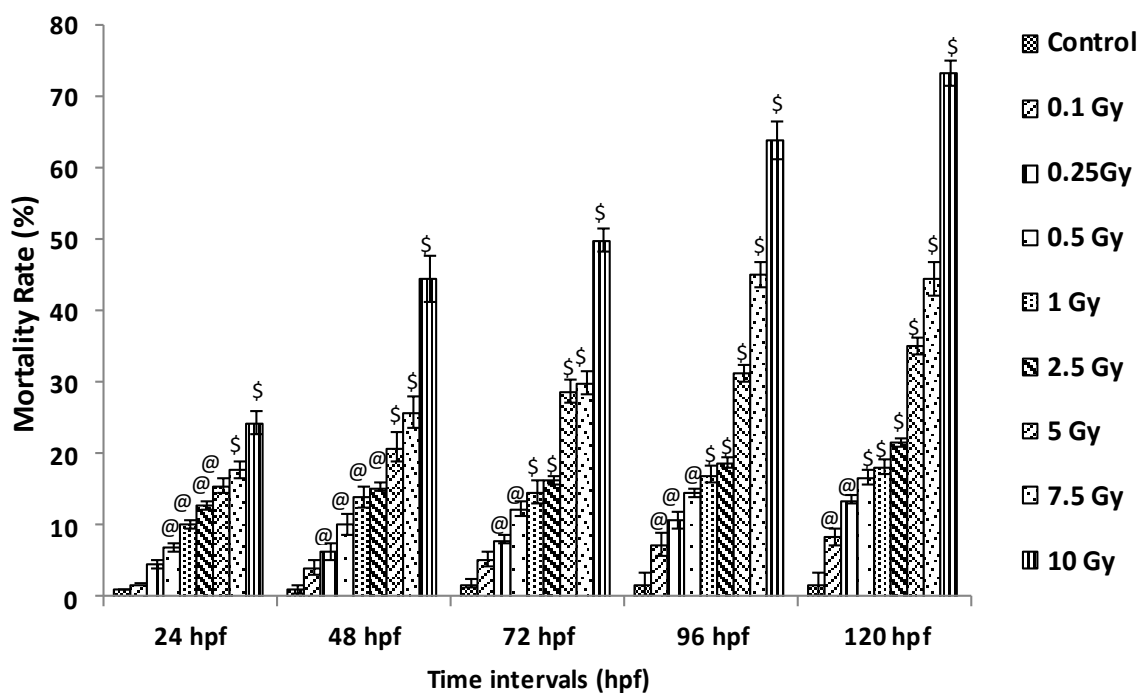|	24 hpf	48 hpf	72 hpf	96 hpf	120 hpf
Control	0±0	45.13±1.61	89.19±3.67	98.26±0	98.26±0.60
0.1 Gy	0±0	20.83±2.09	65.71±3.03	89.93±1.12	89.93±1.02
0.25Gy	0±0	11.45±2.38	61.45±2.24	84.02±1.76	84.72±0.54
0.5 Gy	0±0	9.37±2.07	56.89±2.32	80.20±1.42	81.59±0.87
1 Gy	0±0	7.63±1.59	55.77±7.31	77.77±1.59	80.20±1.04
2.5 Gy	0±0	4.86±1.20	53.57±8.00	72.91±1.04	76.73±0.60
5 Gy	0±0	3.12±1.04	47.48±11.92	58.68±1.59	63.19±1.20
7.5 Gy	0±0	2.43±0.60	45.54±3.04	45.13±1.59	53.81±2.40
10 Gy	0±0	2.08±1.050	21.60±5.66	26.04±3.75	25±1.20
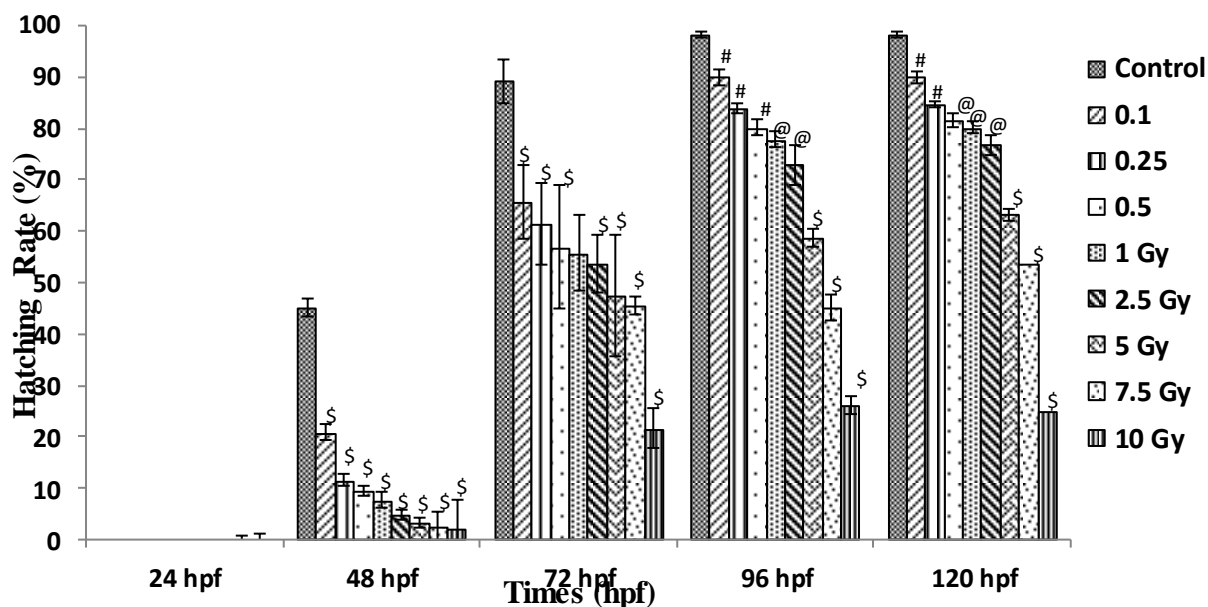
Figure. 3.2: Hatching rate (%) of embryos irradiated with various doses at different time intervals (Mean ± SD). Significant differences between the control and irradiated groups were determined using Chi-square test.

Note: # = $p < 0.05$, @ = $p < 0.01$, \$ = $p < 0.001$

Table 3.4: HT_{50} value embryos irradiated with different doses of gamma radiation (Mean ± SD).

	120hpf
Control	59.90±0.55
0.1 Gy	63.07±1.25
0.25Gy	66.93±1.57
0.5 Gy	66.93±1.15
1 Gy	67.43±0.08
2.5 Gy	68.17±0.77
5 Gy	68.70±0.55
7.5 Gy	69.43±1.15
10 Gy	82.1±2.72
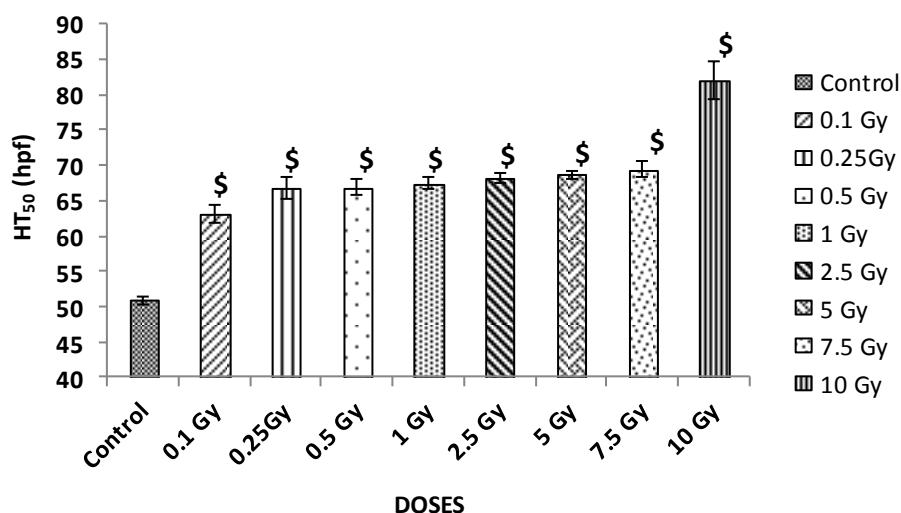
Figure 3.4: HT_{50} value embryos irradiated with different doses of gamma radiation (Mean ± SD). Significant differences between the control and irradiated groups were determined using Student's t-test.

Note: $\$ = p < 0.001$

The hatching rates of zebrafish embryos exposed to various doses of gamma radiation at 120 hpf are represented in Table 3.5 and graphically in Figure 3.5. Embryos showed a dose dependent decrease (F = 702.8, $p < 0.001$) of the hatching rates under laboratory conditions, i.e. the hatching rate decreased (89.93 ± 1.59, 87.72 ± 1.59, 81.6 ± 1.20, 80.21 ± 1.04, 76.74 ± 0.60, 63.19 ± 1.20, 53.82 ± 2.40 and 25 ± 1.80) with increasing doses of gamma radiation 0.1, 0.25, 0.5, 1, 2.5, 5, 7.5 and 10 Gy respectively at 120hpf. Further, the maximum hatching rate (89.91 ± 9.05%) was observed in embryos irradiated with the lowest dose (0.1 Gy) and the minimum (25 ± 9.05%) in embryos irradiated with the highest dose (10 Gy).

Table 3.5: Percentage of control and irradiated embryos hatched by 120 hpf (Mean ± SD).

	120hpf
Control	98.23±0.60
0.1 Gy	89.93±1.59
0.25Gy	84.72±1.60
0.5 Gy	81.6±1.20
1 Gy	80.21±1.04
2.5 Gy	76.74±0.60
5 Gy	63.19±1.20
7.5 Gy	53.82±2.40
10 Gy	25±1.80
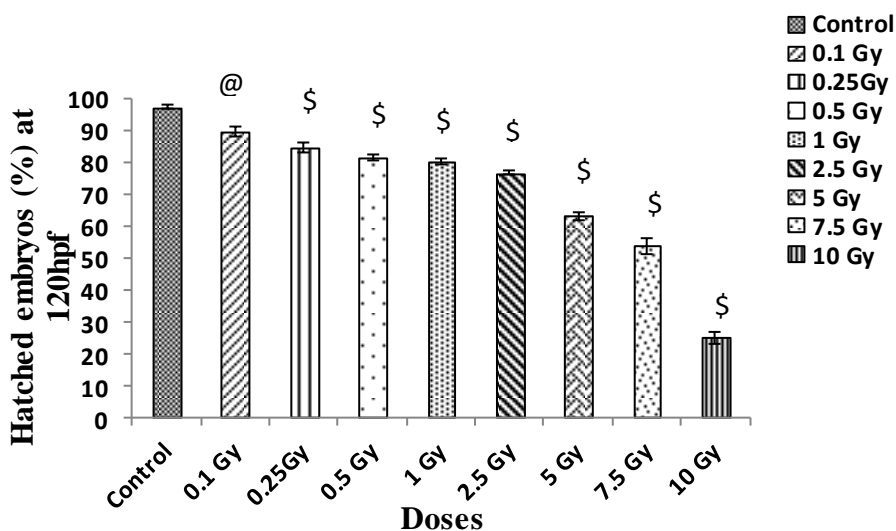
Figure 3.4: Percentage of control and irradiated embryos hatched by 120 hpf (Mean ± SD). Significant differences between the control and irradiated groups were determined using Student's t-test.

Note: @ = $p < 0.01$, \$ = $p < 0.001$

3.3.3. Total Body length:

The size of the irradiated (0.1-10 Gy) and unirradiated (control) larvae attained by 120hpf (as measured by their total body length) are depicted in Figure. 3.6. Irradiated larvae exhibited a dose dependent decrease (F = 5.08, $p < 0.01$) of total body length. Control larvae measured 4.02 ± 0.20 mm, whereas, the irradiated ones ranged from 3.73 ± 0.25, 3.6 ± 0.2, 3.36 ± 0.25, 3.23 ± 0.25, 3.23 ± 0.2, 3.13 ± 0.05, 3.13 ± 0.25 and 3.03 ± 0.50 mm for 0.1, 0.25, 0.5, 1, 2.5, 5, 7.5 and 10 Gy respectively (Table 3.6).

Table 3.6: Total body length in (mm) of control and irradiated larvae (Mean $\pm$ SD) at 120 hpf

	120hpf
Control	4.06±0.20
0.1 Gy	3.73±0.25
0.25Gy	3.60±0.20
0.5 Gy	3.36±0.25
1 Gy	3.23±0.25
2.5 Gy	3.23±0.15
5 Gy	3.13±0.05
7.5 Gy	3.13±0.25
10 Gy	3.03±0.50
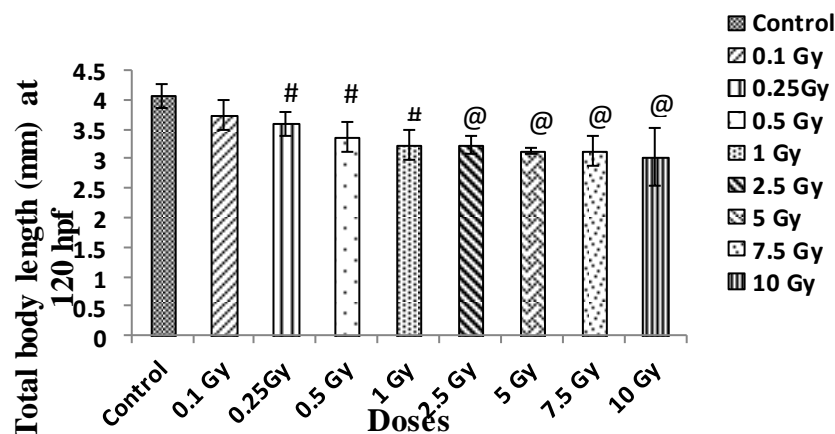
Figure 3.5: Total body length (mm) of control and irradiated larvae (Mean ± SD) at 120 hpf. Significant differences between the control and irradiated groups were determined using Student's t-test.

Note: # = $p < 0.05$, @ = $p < 0.01$

3.3.4. Morphological deformities:

It was clearly observed that high frequency of morphological deformities was induced by various doses of gamma radiation ranging from 0.1-10 Gy in the zebrafish larvae by 120 hpf (Figure 3.7). Malformations such as curved notochord and thin tail were most frequently also observed. Statistically significant (F = 92.62, $p < 0.001$) increase of morphological deformities was seen in larvae irradiated with 0.25 to 10 Gy of gamma radiation compared to the controls. The frequency of malformed larvae ranged from 1.54 ± 0.67, 2.46 ± 0.04, 3.81 ± 1.23, 6.48 ± 1.21, 9.49 ± 1.29, 15.9 ± 3.90, 23.94 ± 4.39 and 58.73 ± 8.42 % in irradiated (0.1, 0.25, 0.5, 1, 2.5, 5, 7.5 and 10 Gy) larvae as compared to the control which showed 0.71 ± 0.61 % (Table 3.7). Further, higher doses of gamma radiation induced severe malformations.

Table 3.7: Frequency (%) of morphological deformities (Mean ± SD) induced by various doses of gamma radiation in zebrafish embryos at 120 hpf

	120hpf
Control	0.69±0.34
0.1 Gy	1.54±0.67
0.25Gy	2.46±0.04
0.5 Gy	3.81±1.23
1 Gy	6.48±1.21
2.5 Gy	9.49±1.29
5 Gy	15.9±3.90
7.5 Gy	23.94±4.39
10 Gy	58.73±8.42
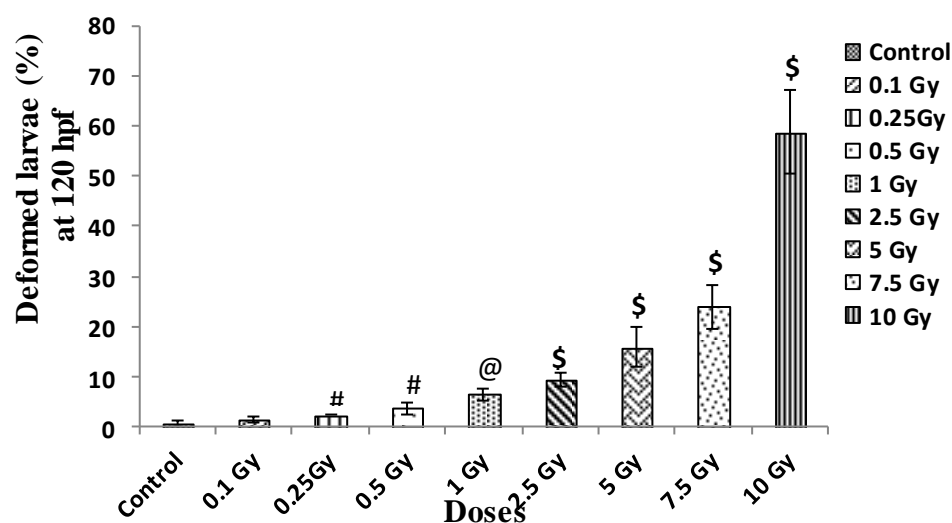
Figure 3.6: Frequency (%) of deformities (Mean ± SD) induced by gamma radiation in zebrafish embryos at 120 hpf. Significant differences between the control and irradiated groups were determined using Student's t-test.

Note: # = $p < 0.05$, @ = $p < 0.01$, \$ = $p < 0.001$

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3.3.5. Comet assay:

The DNA single strand breaks expressed as the mean tail DNA content (% tail DNA) induced by different doses of gamma radiation (0.1-10 Gy) in zebrafish embryo at 24 hpf are given in Figure 3.8. Significant DNA damage (F = 434.9, $p < 0.001$) was observed in all irradiated zebrafish embryos as compared to their controls. Interestingly, a dose dependent increase in the mean % tail DNA was observed with a minimum (16.49 ± 0.66) at the lowest dose (0.1 Gy) and 20.91 ± 0.40, 26.09 ± 0.57, 29.18 ± 1.35, 35.62 ± 0.85, 40.89 ±0.31, 45.73 ± 1.02 and 56.52 ± 0.911 at 0.25, 0.5, 1, 2.5, 5, 7.5 and 10 Gy of radiation (Table 3.8).

Table 3.8: DNA Damage (% of tail DNA) in embryos irradiated with various doses at 24 h after exposure. Data are (Mean ± SD)

	24hpf
Control	7.76±3.00
0.1 Gy	16.49±0.66
0.25Gy	20.91±0.40
0.5 Gy	26.09±0.57
1 Gy	29.18±1.35
2.5 Gy	35.62±0.85
5 Gy	40.89±0.31
7.5 Gy	45.73±1.02
10 Gy	56.52±0.91
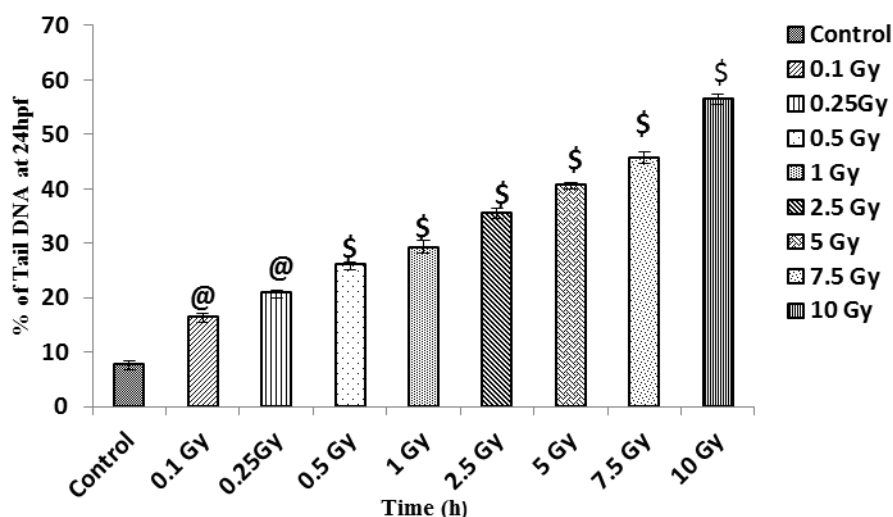
Figure 3.8: DNA Damage (% of tail DNA) in embryos irradiated with various doses at 24 h after exposure. Data are (Mean ± SD). Significant differences between the control and irradiated groups were determined using Student's t-test. Note: @ = $p < 0.01$, \$ = $p < 0.001$

3.3.6. Correlation between Comet assay and Morphological deformities:

The results of the correlation between DNA damage (comet assay) and the morphological deformities induced by irradiation in zebrafish embryos are represented in Figure 3.9. A significant positive relationship ($r = 0.8634$, $p = 0.0027$) was noted between these two parameters.
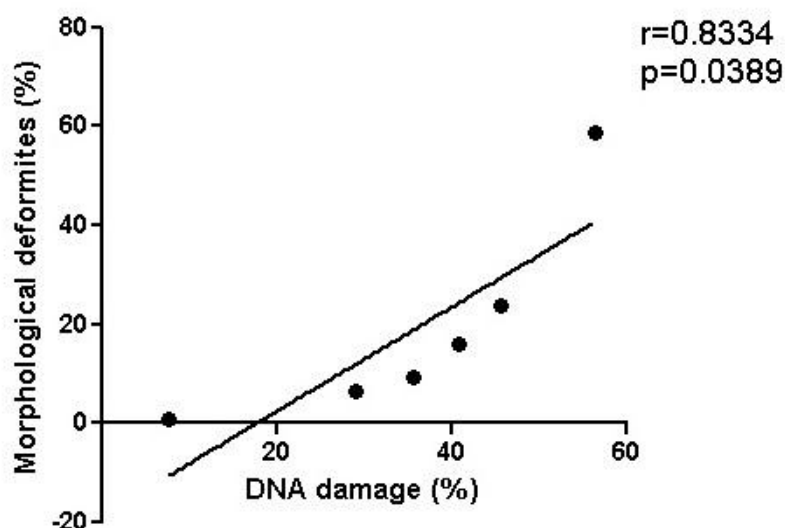
Figure 3.9: Correlation between DNA damage and morphological deformities in zebrafish [Pearson correlation was used and the level of significance was set at 95%

3.4. Discussion:

Toxic effects of gamma radiation on aquatic biota and ecosystems are becoming the emerging concern of recent years. The present study revealed the adverse effects of the acute exposure of gamma radiation in zebrafish embryos as indicated by their increased mortality rate, reduced hatching rate, increased DNA single strand breaks, decreased body length and increased morphological deformities.

3.4.1. Mortality rate:

Increased mortality of irradiated embryos observed in the present study suggests the higher survival risk of the zebrafish larvae upon exposure to gamma rays (toxicity of gamma radiation on zebrafish embryos). A similar increase in mortality rate was reported by Bourrachot et al. (2008) in zebrafish embryos exposed to uranium radioactivity. Sayed and Mitani, (2016) also observed a similar result in *Oryzias latipes* exposed to UV-A radiation. Mortalities were also noted in zebrafish embryos which were exposed to various chemicals (Fraysse et al., 2006) and metals such as copper (Johnson et al., 2007). In the present study, mortality started to occur at a significant level by 24 hpf or day one itself for all the doses studied. This is on par with the observations of McAleer et al. (2005) in which zebrafish embryos exposed to various doses of X-rays at 24 hpf showed increased mortality. However, Freeman et al. (2014) failed to observe a significant increase of mortality and Simon et al. (2011) observed a delay in the mortality of gamma irradiated zebrafish embryos. These may be due to low doses and chronic exposure to gamma radiation which induces a metabolic shift from oxidative phosphorylation to aerobic glycolysis resulting in increased radiation resistance as observed in human cells by Lall et al. (2014). The present

observation of increased mortality in irradiated embryos is supported by additional observations of the present study such as increased DNA damage.

3.4.2. Hatching rate:

Gamma radiation induced a significant delay in hatching as indicated by the increase of the median hatching time of the zebrafish embryos compared to the controls in the present study. Further, a significant decrease of the hatching rate was also noted in the gamma irradiated embryos. Many of the fish hatching strategies depend on the variances in hatching timing, movements and method between the same species developing under natural and hatchery conditions (Korwin, 2011). The decreased hatching rate observed in the present study is in agreement with the findings of Pereira et al. (2011) wherein they observed an impairment of hatching success to zebrafish embryos exposed to gamma radiation. Further, Sayed and Mitani, (2016) also observed reduction in hatching rate of *Oryzias latipes* exposed to UV radiation. Rhee et al. (2012) also observed a decreased hatching rate in the embryos of the fish *Kryptolebias marmoratus* exposed to gamma radiation. This developmental toxicity observed in other fishes may be due to the similar process involved during the hatching process. Further, a linear dose effect of hatchability was observed by Egami et al. (1983) in Medaka fish embryos resulting from the mating between a normal female and an abnormal male with mutation induced by gamma rays. Hatching delay was also observed in a crustacean *Daphnia magna* by Gilbin et al. (2008), in which broods were chronically irradiated with gamma radiation. Similar findings were also reported in zebrafish embryos exposed to chemical or metal toxicants (Fraysse et al., 2006; Johnson et al., 2007; Bourrachot et al., 2008). This may indicate that radiation, chemicals and metals have a similar effect on hatching. However, Freeman et al. (2014) failed to observe significant alternation in hatching rate and

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Simon et al. (2011) observed accelerated hatching time, which may be because of the chronic exposure of 3-24 hpf zebrafish embryos to gamma radiation over a 20-day period in contrast to the acute exposure in our present study. A complex combination of biochemical and physical mechanisms are reported to be involved in the process of hatching in the zebrafish embryos (Inohaya et al., 1997). The chorion is digested by the hatching enzyme (proteolytic enzyme) which is secreted by the hatching gland cells of the embryo. This hatching enzyme contains two constituent proteases: choriolysin H (HCE) and choriolysin L (LCE), which belong to the astacin protease family, a subfamily of zinc-proteases. The delay in embryo development may be a result of derangement of cell division and gene expression by the embryos (Mekkawy et al., 2010). Hatching delay and increase of median hatching time observed in the gamma irradiated zebrafish embryos in our study may be due to the delay / anomaly of the hatching enzyme and / or due to the hypoxia induced by radiological stress.

3.4.3. Body length:

Significant reduction of the body length in the irradiated zebrafish observed in the present study is in line with the observations of Freeman et al. (2014) in which zebrafish embryos were exposed to gamma radiation doses (1, 2, 5, 10 Gy) at 26 hpf. Further, Bourrachot et al. (2008) also reported similar findings in uranium exposed zebrafish. The reduced body length observed in the present study may be because of the radiation stress induced by gamma irradiation. This is supported by our expression studies of the developmental gene (Chapter 4).

3.4.4. Morphological deformities:

High incidence of morphological deformities was induced by various doses of gamma radiation ranging from 0.1-10 Gy in the larvae at 120 hpf. This

increased morphological deformities observed in the present study is on par with the findings of Jing et al. (2017) where they observed morphological abnormalities in zebrafish embryos exposed to ion radiation, such as pericardial sac edema, spinal-column curving and tail curvature. Similarly Torres et al, (2012) observed several developmental abnormalities, mainly posterior/caudal notochord bending/torsion in zebrafish exposed to UV radiation. Further, morphological changes such as yolk sac edema, body curvature, fin blistering, and dwarfism was observed in *Oryzias latipes* exposed to UV radiation (Sayed and Mitani, 2016). Bending of the caudal region was the major kind of morphological deformity noted in the present study. Similar observations were also reported by Pereira et al. (2011) and Freeman et al. (2014) in zebrafish. Zebrafish embryos (midgastrula stage) exposed to UV-A and UV- B radiation showed different kind of deformities such as, spinal deformities, enlarged pericardial sacs, and severe spinal curling or twisting. The degree of damage is directly correlated with the dose of radiation and embryonic stages. Similar deformities were reported by Ishikawa and Hyodo-Taguchi (1997) in medaka (*Oryzias latipes*) exposed to X-rays. The morphological deformities in zebrafish larvae may be mediated by reactive oxygen species (ROS) in addition to DNA damage after exposure to radiation. DNA damage (single / double strand breaks) and change in the expression of *sox2* and *sox19a* gene may also contribute to the induction of morphological deformities (Praveen Kumar et al., 2017).

3.4.5. Genotoxic effects of gamma radiation (Comet assay):

Significant increase of the radiation induced DNA single-strand breaks (% tail DNA) observed in zebrafish embryos in the present study at all the doses studied indicate the genotoxic potential of gamma radiation. Our observation is agreement with Gagnaire et al. (2015), where they reported significant DNA

strand breaks and apoptosis compared to the controls of zebrafish irradiated with 570 mGy/day at 24 hpf. Similar findings were observed in UV irradiated embryos of the African catfish *Clarias gariepinus* (Mekkawy et al., 2010). Increased DNA damage was also reported by Pereira et al. (2011) wherein they observed significant DNA damage in gamma irradiated zebrafish embryonic cells (ZF4). The dose-dependent increase of DNA damage induced by gamma radiation in zebrafish embryos in the present study is on par with the observations of Simon et al. (2011) in which they exposed 6 hpf zebrafish embryos to various doses of gamma radiation (from 1 to 1000 mGy /d). Jarvis and Knowles (2003) also reported a dose dependent increase of DNA damage in zebrafish larvae (5-6 days post laying) exposed to 0.4, 1.2 or 7.2 mGy/h for 1 and 24 h. The DNA damage varies according to the specific radiation (Kielbassa et al. 1997) in addition to their doses, age and species. Irradiation may increase the intracellular levels of hydrogen peroxide and cause oxidative DNA damage, single or double strand breaks (Sayed et al., 2017). The primary products of radiation are rapidly formed free radicals producing biological effects which may last from minutes to years (McFadzen et al., 2000).

Radiation may act either directly on the DNA molecules and induce mutations or indirectly on water molecules to induce water-derived free radicals. These free radicals in turn will react with the nearby molecules in a very short time, resulting in breakage of chemical bonds or oxidation of the affected molecules. The major effect of radiation in cells is DNA strand breaks (Alizadeh et al., 2013).

3.4.6. Correlation between DNA damage and Morphological deformities:

A positive correlation observed in the present study between DNA damage (as represented by comet assay) and morphological deformities in

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zebrafish larvae may suggest the role of DNA single strand breaks in the induction of physical deformities in zebrafish embryos. Similar gamma radiation induced DNA damage and impaired growth were observed in an invertebrate copepod model (Han et al., 2014; Won and Lee, 2014). DNA damage may therefore contribute to all the developmental defects observed in this study.

3.5. Summary:

The major effects of gamma radiation observed in the embryos of zebrafish include decreased hatching rate, increased median hatching time, decreased body length, increased mortality rate, increased morphological deformities and increased DNA damage. This clearly demonstrated the positive mutagenic effect of gamma radiation on zebrafish embryos. These responses indicate that the zebrafish embryo can be used as a sensitive bio-indicator of a genotoxicant within an environmentally realistic range. The alkaline comet assay is an effective technique to assess the genotoxic potential of gamma radiation in whole-organisms. Zebrafish can be a model bio-indicator of radiation exposure in aquatic environments, capable of furnishing good measurable responses to such genotoxicants and mutagenic agents. Further, a positive correlation was noted between DNA damage and morphological deformities during embryo development. Thus, the present study reveals that the DNA damage in the developing embryo could be the possible reasons for the morphological deformities in zebrafish larvae. Thus we may conclude that the above parameters in fish can be used as predictive biomarkers of radioactive contamination in the water bodies and can increase our current understanding of the potential ecotoxicological threats of gamma radiation.

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4.1. Introduction:

Living organisms often get exposed to ionizing radiation from natural sources such as cosmic radiation and natural radionuclides (Oujifard et al., 2015; Gomes et al., 2017). Radiation exposure induces variety of mutations including single strand breaks (SSB), double strand breaks (DSB), chromosomal aberrations and overall genetic instability (Rhee et al., 2012; Han et al., 2014; Song et al., 2014). The various types of DNA damage are induced by ionizing radiation which triggers diverse repair processes. The sensitivity of both tumor cells as well as healthy tissue depend on the cell type, its proliferation and metabolic status (Wu et al., 2014). The sensitivity also depends on the concentration of intracellular scavengers and genetically determined factors (Olive et al., 1990; Breen, 1995; Iannuzzi et al., 2002). Such mechanisms include pathways of DNA repair and signal transduction mechanisms.

The most significant signal transduction pathway responding to DSBs involves the gene ataxia telangiectasia mutated (ATM) (Shiloh, 2003). Ataxia telangiectasia and Rad3-related protein (ATR), in contrast to ATM, responds to single-stranded regions of DNA which are generated at stalled replication forks (Costanzo, 2003). ATR may also be activated at single-stranded regions of DNA exposed during processing of certain DNA damages, such as pyrimidine dimers are generated after ionizing radiation affects. ATM and ATR protein kinases sense DNA damage, activate the DNA damage checkpoint and arrest the cell cycle (Burdak et al., 2008). Such checkpoints include arrest at the G1/S boundary, inhibition of late origin firing (intra-S) and prevention of entry into mitosis (G2/M)

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(Chang et al., 2014). In addition, ATR signaling serves to stabilise stalled replication forks occurred in SSBs. Perhaps the most interesting interplay between the damage response pathways is likely to take place in S phase. Although SSBs and base damage are rapidly repaired, ionizing radiation induces 20-fold higher levels of SSBs relative to DSBs making them a significant lesion. A range of DNA damage response mechanisms operate and help maintain genomic stability in the face of such damage. Increasing evidences suggest that these pathways operate co-operatively. In addition, the relative impact of one mechanism over another most probably depends upon the cell cycle phase and tissue type. Genetic integrity is maintained by an intricate network of DNA repair proteins (Lindahl and Wood, 1999). Defects in this complex machinery are linked with familial predisposition to cancer and other diseases (Bohr, 2002).

Molecular cytogenetic studies on the toxic effects of gamma radiation in Zebrafish and Common carp
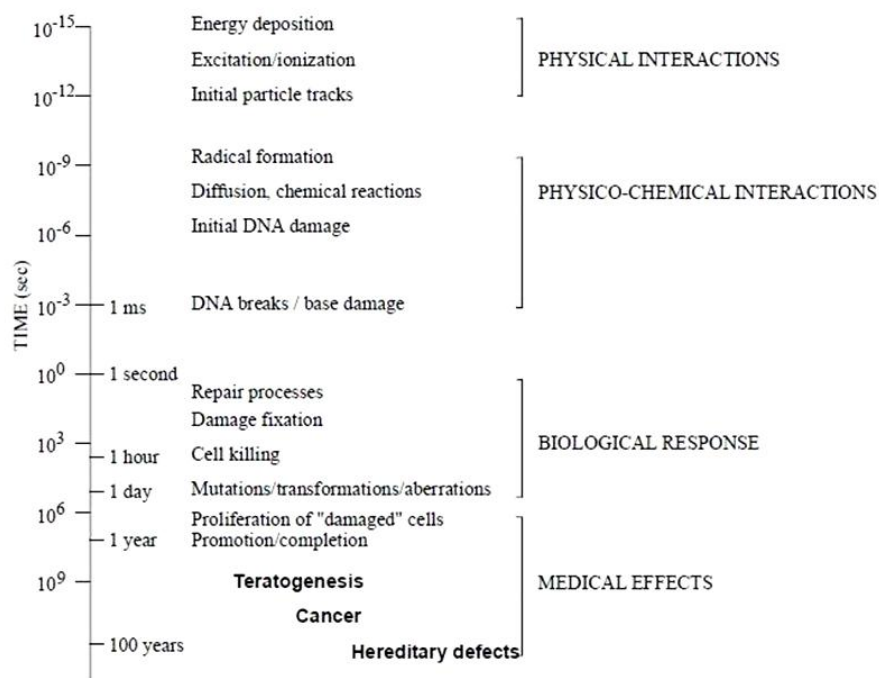
Figure 4.1: Schematics development of the events leading to stochastic radiation effects

4.1.1. Toxocogenomics:

Toxicogenomics is one of the emerging fields in toxicology to identify the genomic signatures of environmental stress/toxicants. The use of gene and protein expression profiles to study the relationship between exposure and disease outcome and understand the interaction of gene–environment and their effect on health (Nuwaysir et al., 1999; Aardema and MacGregor, 2002; Irwin et al., 2004; Jayapal et al., 2010). It has started from early gene-expression studies which described the response of a biological system to a particular toxicant. The combination of classical toxicology study and microarray experiments lead to the development of a new scientific field in 1999 viewed as 'toxicogenomics' which is the merging of major three fields: toxicology, molecular biology and bioinformatics (Nuwaysir et al., 1999). The objectives of toxicogenomics are to understand the relationship between gene–environment interaction and disease susceptibility; to discover useful biological response markers of disease and exposure to toxic substances; and to sort out the molecular mechanisms of toxicity (Nuwaysir et al., 1999; Jayapal et al., 2010).

The use of toxicogenomic approaches to biomarker discovery can be widely applied to both environmental and clinical exposure scenarios in response to disease (Brown et al., 2004; Benninghoff and Williams, 2007). Further, they can serve as quantitative measures of toxicant exposures and biologically effective

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doses, as well as early warning signals of biologic effect. Ideally, biomarkers can be identified at any stage along the disease continuum, from external exposure to the final response of interest or concern. Exposure and disease outcome specific patterns of gene profiles have been used to identify molecular changes that can be used as biomarkers of toxicity (Hamadeh et al., 2004) and these profiles can provide insights into mechanisms of toxicity (Fertuck et al., 2003). When the body's cells are exposed to a toxicant, they respond by altering the pattern of expression of genes within their chromosomes. The production of protein encoded by a given gene may be over expressed, decreased, or remain unchanged, depending upon the type of exposure. These events could be identified when a chemical/physical toxicant interactions with organisms at the cellular and molecular levels leading to a toxic endpoint.

Applications of toxicogenomics:

- ➢ Reveal mechanisms of action of environmental toxicant through the recognition of the pattern of gene expression
- ➢ Identify and catalog the genomic expression fingerprint of a particular toxicant serving as a biomarker to assess human exposure
- ➢ Examine responses to chronic low doses of toxicants or environmental pollutants
- ➢ Helps clarifying whether a specific toxicant signature is altered depending upon the stage in the developmental process or has a defined health condition
- ➢ Infer effects of toxicants from one species to another

➢ Discover toxicants on the basis of tissue specific patterns of gene expression by establishing reference molecular signatures for exposure of specific toxicant (chemical/physical)

➢ Helps in finding alternates of safety for use in drug trials

➢ Elucidated the interactions of complex chemical mixtures (Jayapal et al., 2010)

4.1.2. Biomarkers:

Biomarkers are valuable tools for understanding the nature and extent of human exposure and risk from environmental toxicants. Biological effects elicited by environmental pollutants can be detected at various levels. It is known to act through direct or indirect interaction with the DNA molecule, leading to DNA damage. DNA repair activities are extremely important because unrepaired DNA damage has the potential to be mutagenic, cytotoxic and carcinogenic. Collectively, the response systems to DNA damage that reduce the yield of mutations and chromosomal aberrations are known as DNA repair systems (Powell et al., 2005). This complex cellular system acts at three levels: (a) arresting the cell cycle to allow time for DNA repair; (b) triggering the signal transduction events to activate the repair components; and (c) directly reversing, excising or tolerating DNA damage via constitutive and induced activities (Begley and Samson, 2004). If DNA damage is not repaired, cells undergo complex enzymatic reactions that might lead to apoptosis, necrosis or other forms of cell death (Nyberg et al., 2002; Su, 2006). Several different types of lesions could be produced in the DNA molecule, different pathways for DNA repair are known, each one being specific for a type of lesion

(Hoeijmakers, 2001). Repair of damaged DNA is a continuous process in organisms and is initiated by transcription of genes that are induced in response to specific types of DNA damage. A strand break can occur in one or in both strands, and, depending on the type of breakage, different repair mechanisms will be induced to resolve the DNA damage and reduce breaks within damaged cells (Dhawan et al., 2009). Once DNA damage such as strand breaks are detected, the cell cycle is arrested and DNA repair is initiated (Bladen et al., 2005). A series of proteins mediate damage recognition, signaling, end processing, and end re-joining, via either non-homologous end-joining (NHEJ) or homologous recombination (HR) pathways (Kobayashi et al., 2008). When the homologous sister ends of the broken strands are in close proximity, repair using the intact sister template can be straightforward and accurate through the HR pathway (Sonoda et al., 2006). Strand breaks (single and double) are a common type of DNA damage that is repaired predominantly through non-homologous end-joining or homologous recombination. The tumor suppressor protein p53 has a central role in cellular stress responses. It elicits its normal functions mainly by acting as a transcription factor, regulating the transcription of genes involved in cell cycle arrest (e.g. *p21*, *Gadd45ᵨ*, *CyclinG1*), DNA repair (e.g. *XPC*, *DDB2*) and apoptosis (e.g. *Bcl-2*, *Bax*) (Kohn, 1999; Ford, 2005; Abumani and ….2016; sayed 2017; jing 2017).
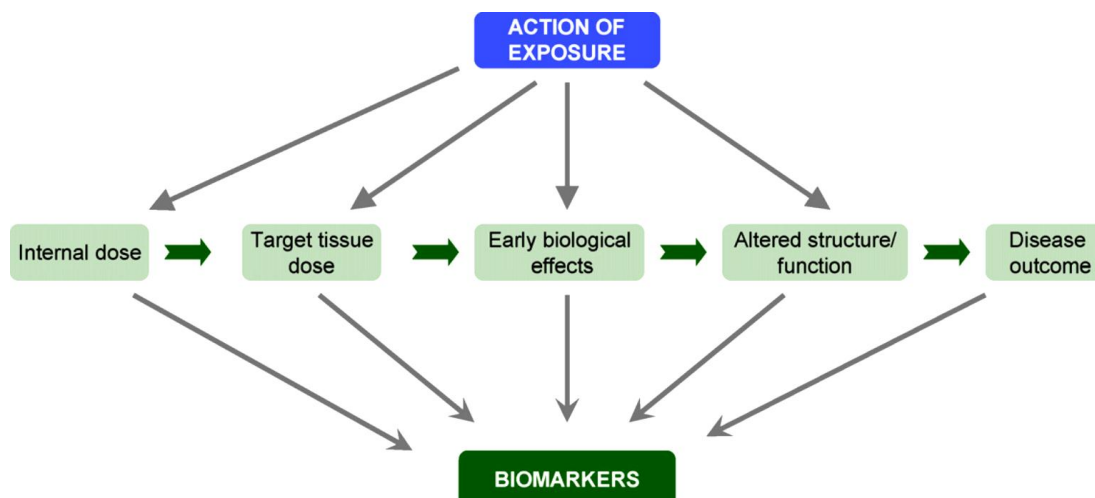
Figure 4.2: Schematics representation of action of exposure

4.1.3. Toxic effect of radiation on gene expression:

The measurement of gene expression levels before and after exposure to a mutagen/chemical/physical agent can both provide information about the mechanism of action of toxicants as well as form a "genetic signature" from the pattern of gene expression (Jaafar et al., 2013; Freeman et al., 2014). Few researchers have addressed change in the expression of DNA repair and developmental gene in fish and fish embryo (Lyng et al., 2004; Dowd et al., 2006; Smith et al., 2007; Salbu et al., 2008; Rhee et al., 2012; Jaafar et al., 2013; Freeman et al., 2014; Song et al., 2014; Anbumani and Mohankumar, 2016; Sayed et al., 2017; Jing, et al., 2017). However, studies on expression profile of genes involved in fundamental physiological function are scanty. Repair of DNA damage is initiated by induction of genes that code for products that drive the repair process. Key genes involved in NHEJ are *xrcc5*, *xrcc6* (encoding for Ku80 and Ku70 protein, respectively), and *rad51* is critical for HR repair (Thacker and Zdzienicka,

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2004). These genes involved in NHEJ and HR repair are induced after radiation (Bladen et al., 2007a, b; Sandrini et al., 2009b) as well as after metal exposure (Gonzalez et al., 2006; Sandrini et al., 2009a) in response to induction of strand breaks. *In vitro* studies in zebrafish reported induction of *xrcc5* 12 h after exposure to UV-B (Sandrini et al., 2009b) and 24 h after exposure to copper (Sandrini et al., 2009a). In adult zebrafish, a 10-fold induction of *rad51* expression was reported in liver after 63-day dietary exposure to methylmercury (Gonzalez et al., 2005), a 32-fold induction in expression after 7 day exposure to cadmium (Gonzalez et al., 2006), and a 5-fold peak in expression in gills after 8 day exposure to copper (Lerebours et al., 2009). It has been suggested that single strand breaks occur rapidly and are rapidly repaired too compared with the more complex double-strand breaks (Brendler-Schwaab et al., 2005), but the timing of induction of damage and expression of these repair genes has not been established.

Bax is a pro-apoptotic member that exerts its function by homodimerizing with each other and accumulating in the mitochondrial membrane (Miyashita et al., 1994). It has been shown that transcription factor p53 affects cell cycle arrest or apoptosis in response to a variety of genotoxic and physical stresses (Goloudina et al., 2012). Apoptosis may be elicited, in part, by p53- stimulated transcription of the pro-apoptotic gene Bax and repression of the anti-apoptotic gene Bcl-2 (Xin and Deng 2006; Zhao et al., 2012). Rad51 genes are involving in the homologous recombination (HR) DNA repair mechanisms (Hoeijmakers, 2001). Rad proteins bind to broken ends, locate the appropriate homologous sequence, and recruit intermediates for DNA synthesis and final ligation (Takata et al., 2000; Kobayashi

et al., 2008). However, in tightly packed chromatin, close proximity of homologous template is not always possible, and NHEJ is suggested as the predominant strand break repair pathway in vertebrate cells (Takata et al., 1998). NHEJ involves formation of Ku80/Ku70 heterodimer that binds the free ends of broken DNA strands, spanning the gap and recruiting ligating enzymes to re-join the strands (Gu et al., 1997; Jones et al., 2001; Kobayashi et al., 2008). Large-scale tools in risk assessment of pollution have been increasingly recognized (Rowlands et al., 2014). *Sox* genes play an important role in multitude of developmental and physiological processes (Zhen and Wan, 2015; Yin, 2017). The Sox proteins act as transcriptional activators, transcriptional repression and architectural roles (Michael, 2010). The major functions of these genes include skeletogenesis (Smits et al., 2001), stem cell development in the embryo (Avilion et al., 2003), cardiogenesis (Akiyama et al., 2004; Andrew,et al., 2017), neurogenesis (Pevny and Placzek, 2005), sex determination (Polanco et al., 2007) and hematopoiesis (Schilham et al., 1997) in zebrafish. *Sox*2 gene has diverse obligatory roles to play in zebrafish embryonic development (Okuda et. al., 2010; Pavlou et al., 2014). *Sox*2 gene encodes a transcriptional factor and is well known for its role in maintaining pluripotent stem cell population and differentiation during early development. The *sox19* gene play very important role in during embryogenesis and functions in morphogenesis and differentiation of cartilage and bone (Ma et al., 2017). Abnormality in the tail region and central nervous system observed in zebrafish embryo when *sox2*, *sox3* and *sox19a* were knocked down (Okuda et al., 2010). Water temperature is affected

the gene expression of *Sox5, Sox8*, and *Sox9* at the early stage of development of golden pompano larvae (Ma et al., 2017).

Zebrafish (*Danio rerio*) is an organism commonly used as a biological model in several toxicological studies (Amanuma et al., 2000; Seok et al., 2006 Craig et al., 2007; Pomati et al., 2007). Besides characteristics like easy handling in laboratory, low maintenance costs and sequenced genome, this species has demonstrated similarities in many physiological processes with mammals (Stern and Zon, 2003; Grabher and Look 2006; Lam et al., 2006).

4.2. Materials and methods:

The objectives of this study were to evaluate changes in the expression of key genes involved in DNA repair and development of zebrafish.

4.2.1. Experimental specimen fish:

Adult zebra fish *(Danio rerio)* were procured from Aquaculture farm, Margao, Goa. The fish were acclimatised for one month in a glass aquarium of 50 litre capacity fitted with aerators and heaters at 28±1ºC with 14:10 light: dark photoperiod. Water was manually renewed by changing 50% of the total volume once in every week and by refilling the evaporated water every day. Fishes were fed twice daily with live brine shrimps (*Artemia salina*) and fish feed. The pH, dissolved oxygen content and total hardness of the aquarium water were analysed by standard methods.

4.2.2. Acclimatization in aquaria

Prior to irradiation, fingerlings of length 4 ± 1.0 cm and weight between $1 -$ 0.95 g were acclimatized to laboratory conditions in glass aquaria for 2 weeks (temp. $26.2 \pm 2.03°C$, 12-h light/dark cycle, pH 7.1 ± 0.25, DO 8.4 ± 1.1 mg/L). These fishes were fed with oil cake. The water was changed once a day.

4.2.3. Egg production:

As and when the eggs were needed for studies, adult male and female fish (ratio = 1:2) were placed in a hatching box (tray) in the late evening of the previous days and allowed to remain together overnight for spawning. Spawning process was triggered in the early morning hours of the day by putting on the lights which lasted around one hour. Viable eggs were collected and rinsed at least three times with E3 medium. E3 medium was prepared by dissolving various salts in the standard hatchery water as per Brand et al. (2002) (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$) with pH 7.2–7.3, dissolved oxygen 6.3 mg/L, total hardness 65 mg/L (as $CaCO_3$) and temperature $28 \pm 1°C$. All the chemicals used were of analytical grade. In order to ensure developmental synchronization at the beginning of exposure of gamma radiation, the embryos at the blastula stage of about 3 hours post fertilization (hpf) were sorted under a stereo microscope and employed for irradiation.

4.2.4. Exposure to radiation:

4.2.4.1. Zebrafish adult:

Adult fishes (10 animals per group) were exposed to a whole body irradiation of a single dose of 5 Gy allowing 0.5 cm deviations at the center of the dose rate using a gamma radiation source of a Cobalt Teletherapy Unit at Goa

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Medical College, Goa. Unirradiated fishes maintained in par with the experimental fishes served as controls. Blood samples were collected from the irradiated fishes as well as their respective unirradiated controls at 24, 48 and 72 h of irradiation.

4.2.4.2. Zebrafish embryos:

The assay was mainly based on the embryo test procedure developed by OECD guideline, 2013. In brief, embryos were transferred into a 96-well multiplate, one embryo per well filled with E3 medium. Embryos (3hpf) were irradiated with one time for the appropriate length of time based on dose rate on day of dosing (acute exposure) of 5 Gy of gamma rays from a Co_{60} teletherapy unit at Goa Medical College, Goa. Parallel control groups were mock irradiated by placing the samples in the irradiator. Both the control and irradiated embryos were maintained at $28 \pm 1°C$ with a 14:10 hrs (light: dark) photoperiod. E3 media were renewed regularly at 24 h intervals of time.

4.2.5. Sample collection from fish:

Different samples (blood, gill and muscle) were collected from both irradiated and control fish at various time intervals (24, 48 and 72h). Zebrafish embryos (20 each) in triplicate at 24, 72 and 120 hpf from both irradiated and control zebrafish embryos.

4.2.6. Gene expression study:

4.2.6.1. RNA extraction and cDNA synthesis:

Gene expression analysis was performed by quantitative polymerase chain reaction (qPCR), as described by Chelly et al. (1988). Total RNA was isolated from both control and exposed zebrafish adult and embryos using TRIzol reagent

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(Invitrogen), following the manufacturer's recommendations. RNA was eluted into 30 μl, the quality and quantity of the RNA was evaluated by NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). All samples were diluted to 500 ng/μl total RNA, and First-strand cDNA molecules were synthesized from 500ng of total RNA using the SuperScript III Reverse Transcriptase (Invitrogen, USA) according to the manufacturer's instructions. cDNA was stored at -80 ^{0}C until qRT-PCR gene expression analysis.

4.2.6.2. Selection of primers for DNA repair genes:

Primers were selected by Primer Blast (National Center for Biotechnology Information, NCBI). The selected primers were checked using DNA calculator (Sigma-Aldrich) and OligoCalc (Northwestern University, USA) to avoid self-annealing, complementarity, potential hairpins and secondary structure. Performance and amplicon size was confirmed on a 2 % agarose gel after PCR amplification. Primer details are listed in Table 4.1.

Table 4.1: Details of target genes for q-PCR along with primers

Gene name	Accession ID	Primer sequence	Function	Reference
*sox*2	NM_213118.1	F: CTCGGGAAACAACCAGAAAA R: TCGCTCTCGGACAGAAGTTT	Maintenance of embryonic and neural stem cells.	Desai et al. (2011)
sox19a	NM_130908.1	F: TGTCAACAGCAACAACAGCA R: GTTGTGCATTTTGGGGTTCT	Development of central nervous system	
β actin	NM_181601.3	F: CGAGCTGTCTTCCCATCCA R: TCACCAACGTAGCTGTCTTTCTG	Housekeeping genes	
p53	AF365873	F:GGGCAATCAGCGAGCAAA R: ACTGACCTTCCTGAGTCTCCA	Growth arrest, DNA repair and apoptosis	Gonzalez et al. (2005)
bax	AF231015	F:GGCTATTTCAACCAGGGTTCC R: TGCGAATCACCAATGCTGT	Apoptosis	
rad51	BC062849	F:TGCTGCGTCTCGCTGA R: GCCTCGGCCTCTGGTAA	DNA repair	

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4.2.6.3. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR):

Real-time PCR reactions were carried out with one cycle at 95 °C for 10 min and 40 amplification cycles at 95 °C for 10 s, 58 °C for 15 s and 72 °C for 15 s). Reaction mixture (20 µL) contained 4 µL of reverse transcribed product (cDNA) as template, 10 µL of SsoFast EvaGreen supermix (Bio-Rad, USA), 1 µL each gene specific primer at a final concentration of 300 nM and 4 µL RNase free water. The amplification reaction was carried out using CFX connect Real-Time PCR Detection System (Bio-Rad, USA). The *β actin* gene was selected as reference gene for the present study, due to its high stability (McCurley et al., 2008). Further, the stability of the *β actin* towards gamma radiation was confirmed by experimentation. There was no statistically significant difference for expression of *β-actin* between control and exposed groups at each time interval. However, therefore, *β actin* was used as reference gene for normalization. Relative gene expression of *p53*, *bax* and *rad51* in adult zebrafish, *sox*2, *sox19a and p53* in zebrafish embryos were compared to the reference gene, *β actin* and were determined using CFX Manager™ software (Deepa et al., 2013).

4.2.7. Statistical analysis:

Statistical analyses were performed using IBM SPSS 23 software package. Data of relative expression were analyzed using student's t-test. For gene expression analysis, standard curve for each plate was carried out and the efficiency of qPCR was calculated. Samples from the same experiment run over multiple plates were adjusted to the plate with the efficiency closest to 1 by resolving for slope and intercept of the standard curve. One way analysis was

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carried out for time dependent gene expression. Two-way analysis of variance was used to test the influence of dose and time and their interaction on gene expression. A level of probability of $p < 0.05$ was considered as a statistically significant data.

4.3. Results:

4.3.1. Gene expression in zebrafish:

Genes analyzed in the present study are related to DNA repair system and could be grouped according to their function: cell cycle arrest (*p53*), apoptosis (*bax*) and DNA repair (*rad51*). Gamma radiation effects on gene expression were more evident in zebrafishes exposed to 5 Gy. Statistically significant increase in the expression of *p53, bax and rad51* genes were observed in the different tissues of zebrafish irradiated as compared to the unirradiated fishes.

The expression of *p53* was significantly up-regulated in various tissues of gamma irradiated fish compared to control at all the time intervals. The *p53* gene expression level in blood was 2.74 ± 0.28, t-test, p<0.001 at 24 h, 2.63 ± 0.01, t-test, p<0.001 at 48 h and 1.35 ± 0.003, t-test, p<0.05 at 72 h. The *p53* gene expression level in gill tissue was at 24 h (2.61 ± 0.02, t-test, p<0.001), at 48 h (1.85 ± 0.05, t-test, p<0.001) and at 72 h (1.44 ± 0.08, t-test, p<0.01).The *p53* gene expression in muscle tissue was at 24 h (1.84 ± 0.007, t-test, p<0.001), at 48 h (1.47 ± 0.006, t-test, p<0.01) and at 72 h (1.06 ± 0.003, t-test, ns). Further, One way ANOVA showed significance different between the time intervals in blood (F= 68.45; $p< 0.001$; table 4.2), gill tissue (F= 341.1; $p< 0.001$; table 4.3) and muscle tissue (F= 148950; $p< 0.001$; table 4.4). The Two way ANOVA was carried out to see the expression of *p53* gene in different tissues and different time intervals (Table 4.5). Significant variation was observed with tissue (F=

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145.13; $p< 0.001$), time intervals (F= 293.84; $p< 0.001$), as well as the interaction between tissue and time (F= 23.03; $p< 0.001$).

Figure 4.3: Effect of gamma radiation on *p53* gene expression in zebrafish blood. Each value is expressed as the Mean ± S.D. Significant differences between the control and irradiated groups were determined using Student's t-test.

Note: ***= P<0.001, ** = P<0.01, * = P<0.05

Table 4.2: One way ANOVA of *p53* gene expression in zebrafish blood

ANOVA Table	SS	df	MS	*F*	P-value
Treatment (between columns)	2.115	2	1.057	68.45	p<0.001
Residual (within columns)	0.0186	6	0.0031		
Total	2.133	8			

Figure 4.3: Effect of gamma radiation on *p53* gene expression in zebrafish gill tissue. Each value is expressed as the Mean ± S.D. Significant differences between the control and irradiated groups were determined using Student's t-test. Note: ***= P<0.001, ** = P<0.01.

Table 4.3: One way ANOVA of *p53* gene expression in zebrafish gill tissue

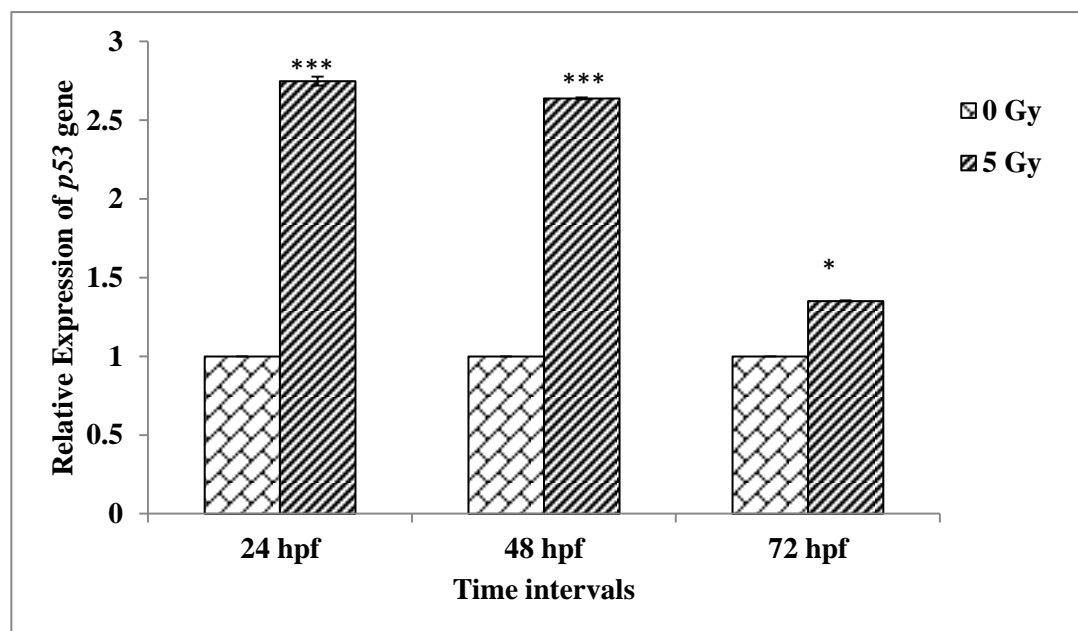| ANOVA Table	SS	df	MS	*F*	P-value
Treatment (between columns)	2.115	2	1.057	341.1	p<0.001
Residual (within columns)	0.0186	6	0.0031		
Total	2.133	8			

Figure 4.4: Effect of gamma radiation on *p53* gene expression in zebrafish muscle tissue. Each value is expressed as the Mean ± S.D. Significant differences between the control and irradiated groups were determined using Student's t-test. Note: ***= P<0.001, ** = P<0.01, * = P<0.05

Table 4.4: One way ANOVA of p53 gene expression in zebrafish muscle tissue

ANOVA Table	SS	df	MS	*F*	P-value
Treatment (between columns)	0.9134	2	0.4567	148950	p<0.001
Residual (within columns)	0.000188	6	0.00003133		
Total	0.9136	8			

Table 4.5: Two way ANOVA of p53 gene expression in different tissue (blood, gill and muscle tissue) of zebrafish.

Source	Type III Sum of Squares	df	Mean Square	F value	Significance
Tissue	2.836	2	1.418	145.133	0.001
Time	5.742	2	2.871	293.848	0.001
Tissue * Time	0.900	4	0.225	23.031	0.001

Note: $r^2 = .982$

The *bax* gene expression was also up-regulated in all the tissues of the gamma irradiated at various time intervals. The expression of *bax* gene in blood was at 24 h (2.65 ±0.058, t-test, p<0.001), 48 h (2.16 ± 0.024, t-test, p<0.001) and 72 h (1.39 ±0.054 t-test, p<0.05). The expression of bax gene in gill tissue was at 24 h (2.44 ±0.134, t-test, p<0.001), 48 h (2.27 ±0.024, t-test, p<0.001) and 72 h (1.74 ±0.042, t-test, p<0.01).The *bax* gene expression in muscle tissue was at 24 h (1.64 ±0.014, t-test, p<0.01), 48 h (1.74 ±0.016, t-test, p<0.01) and 72 h (1.76 ±0.021, t-test, p<0.05). Further, One way ANOVA showed significance different between the time intervals in blood (F= 529.6; *p*< 0.001) Table 4.6, gill tissue (F= 59.11; *p*< 0.001) Table 4.7 and muscle tissue (F= 41.66; *p*< 0.001) Table 4.8. The Two way ANOVA was carried out to test the influence of time and tissue type on *bax* gene expression (Table 4.9). Significant variation of *bax* gene expression was observed between tissues (F= 228.41; *p*< 0.001), time intervals (F= 369.53; *p*< 0.001) and the interaction between tissue and time (F= 51.68; *p*< 0.001).
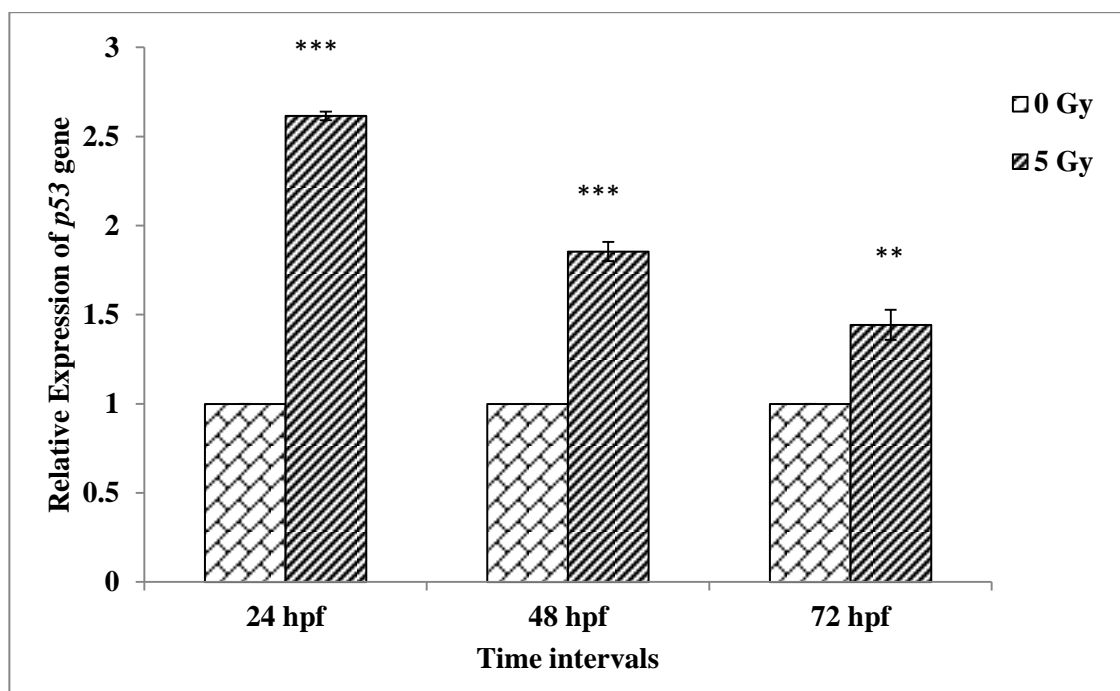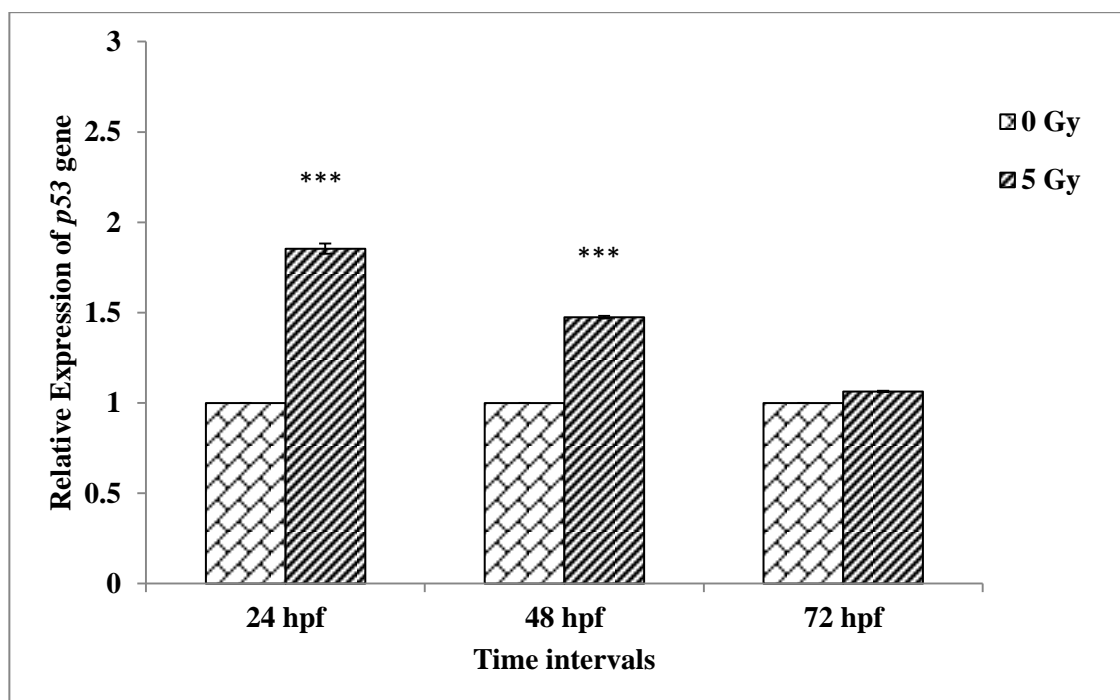
Figure 4.5: Effect of gamma radiation on *bax* gene expression in zebrafish blood. Each value is expressed as the Mean ± S.D. Significant differences between the control and irradiated groups were determined using Student's t-test.

Note: ***= P<0.001, ** = P<0.01, * = P<0.05

Table 4.6: One way ANOVA of *bax* gene expression in zebrafish blood

ANOVA Table	SS	df	MS	*F*	P-value
Treatment (between columns)	2.421	2	1.210	529.6	p<0.001
Residual (within columns)	0.01371	6	0.002285		
Total	2.434	8			

Figure 4.6: Effect of gamma radiation on *bax* gene expression in zebrafish gill tissue. Each value is expressed as the Mean ± S.D. Significant differences between the control and irradiated groups were determined using Student's t-test. Note: ***= P<0.001, ** = P<0.01, * = P<0.05

Table 4.7: One way ANOVA of *bax* gene expression in zebrafish gill tissue

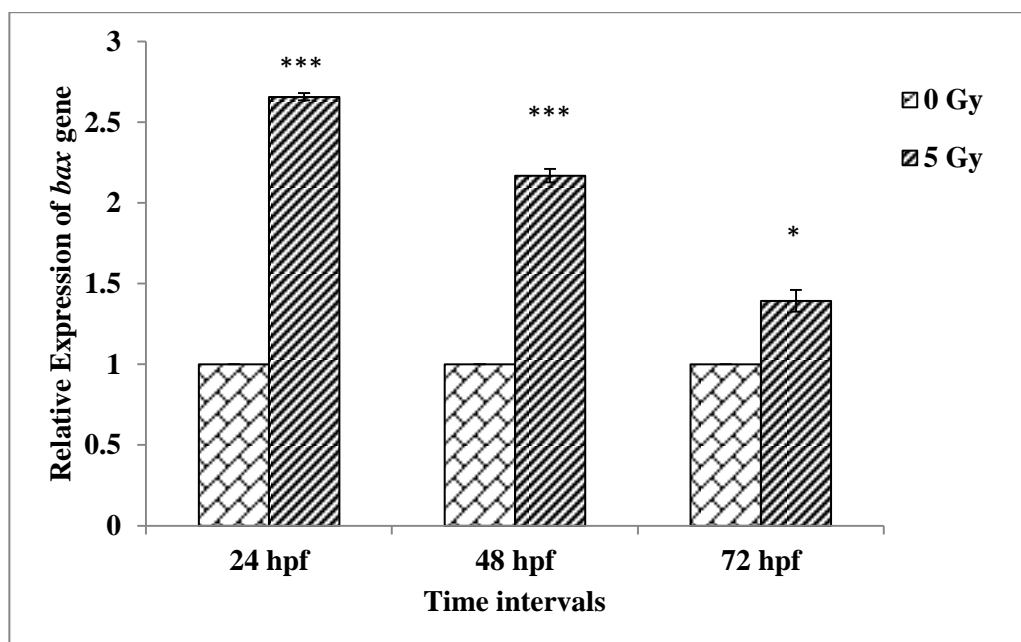| ANOVA Table	SS	df	MS	*F*	P-value
Treatment (between columns)	SS	df	MS	59.11	p<0.001
Residual (within columns)	0.7998	2	0.3999		
Total	0.04059	6	0.006765		

Figure 4.7: Effect of gamma radiation on *bax* gene expression in zebrafish muscle tissue. Each value is expressed as the Mean ± S.D. Significant differences between the control and irradiated groups were determined using Student's t-test. Note: ***= P<0.001, ** = P<0.01, * = P<0.05

Table 4.8: One way ANOVA of *bax* gene expression in zebrafish muscle tissue

ANOVA Table	SS	df	MS	*F*	P-value
Treatment (between columns)	0.0248	2	0.0124	41.66	p<0.001
Residual (within columns)	0.001786	6	0.0002977		
Total	0.02659	8			

Table 4.9: Two way ANOVA of *bax* gene expression in different tissue (blood, gill and muscle tissue) of zebrafish

Source	Type III Sum of Squares	df	Mean Square	F	Significance
Tissue	1.670	2	.835	228.411	0.001
Time	2.702	2	1.351	369.538	0.001
Tissue * Time	0.756	4	0.189	51.681	0.001

The *rad51*gene expression was also up-regulated in various tissues of the gamma irradiated fish at different time intervals. The *rad51* gene expression in blood was at 24 h (1.32 ±0.024, t-test, p<0.05), 48 h (2.09 ±0.054, t-test, p<0.001) and 72 h (2.59 ±0.084, t-test, p<0.001). The *rad51* gene expression in gill tissue was at 24 h (2.32 ±0.021, t-test, p<0.001), 48 h (2.66 ±0.053, t-test, p<0.001) and 72 h (2.59 ±0.082, t-test, p<0.001).The expression of *rad51* gene in muscle tissue was at 24 h (1.56 ±0.007, t-test, p<0.001), 48 h (2.14 ±0.006, t-test, p<0.001) and 72 h (1.42 ±0.003, t-test, p<0.001). Further, One way ANOVA showed significance different between the time intervals in blood (F= 936.6; $p<$ 0.001; Table 4.10), gill tissue (F= 88.28; $p<$ 0.001; Table 4.11) and muscle tissue (F= 139500; $p<$ 0.001; Table 4.12). Significant variation was observed between tissue type (F= 593.52; $p<$ 0.001), time intervals (F= 338.95; $p<$ 0.001), and tissue and time interaction (F= 175.22; $p<$ 0.001) by the two-way ANOVA (Table 4.13).
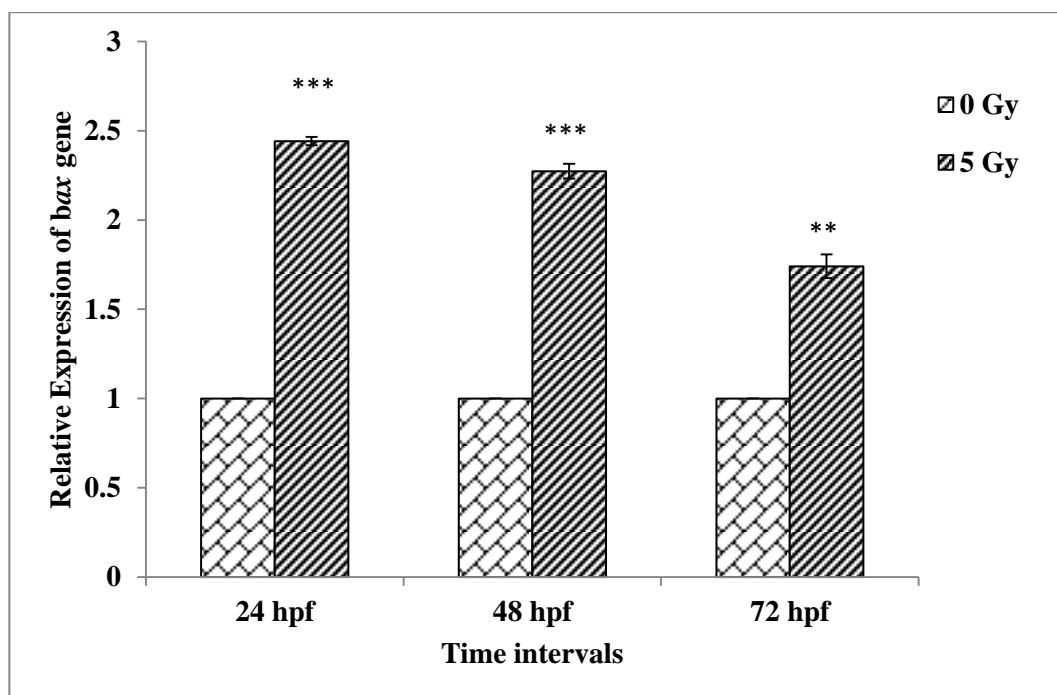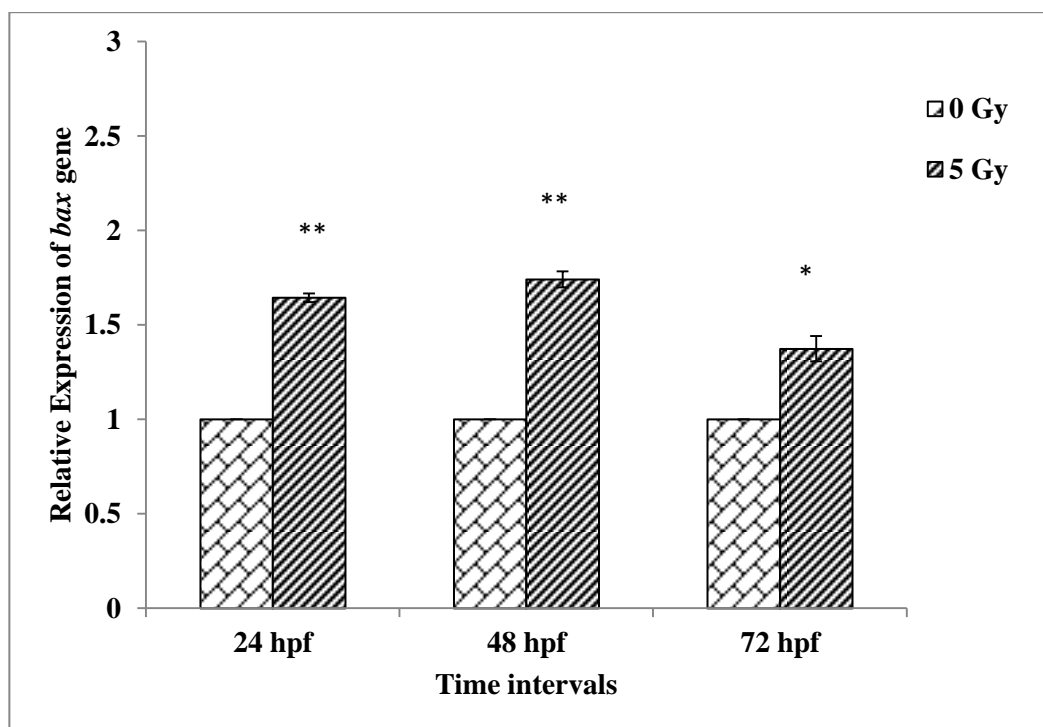
Figure 4.8: Effect of gamma radiation on *rad51* gene expression in zebrafish blood. Each value is expressed as the Mean ± S.D. Significant differences between the control and irradiated groups were determined using Student's t-test. Note: ***= P<0.001, ** = P<0.01, * = P<0.05

Table 4.10: One way ANOVA of *rad51* gene expression in zebrafish blood

ANOVA Table	SS	df	MS	*F*	P-value
Treatment (between columns)	2.456	2	1.228	936.6	p<0.001
Residual (within columns)	0.007866	6	0.001311		
Total	2.464	8			

Figure 4.8: Effect of gamma radiation on *rad51* gene expression in zebrafish gill tissue. Each value is expressed as the Mean ± S.D. Significant differences between the control and irradiated groups were determined using Student's t-test. Note: ***= P<0.001, ** = P<0.01, * = P<0.05

Table 4.11: One way ANOVA of *rad51* gene expression in zebrafish gill tissue

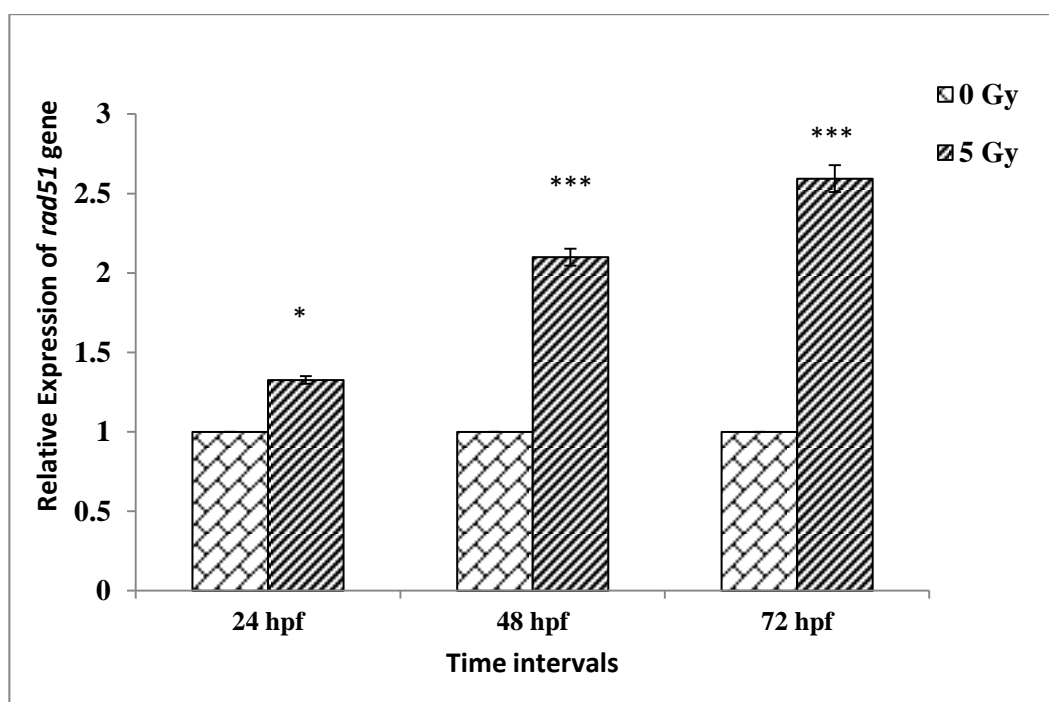| ANOVA Table	SS	df	MS	*F*	P-value
Treatment (between columns)	0.1934	2	0.09670	88.28	p<0.001
Residual (within columns)	0.006572	6	0.001095		
Total	0.2000	8			

Figure 4.9: Effect of gamma radiation on *rad51* gene expression in zebrafish muscle tissue. Each value is expressed as the Mean ± S.D. Significant differences between the control and irradiated groups were determined using Student's t-test. Note: ***= P<0.001, ** = P<0.01, * = P<0.05

Table 4.12: One way ANOVA of *rad51* gene expression in zebrafish muscle tissue

ANOVA Table	SS	df	MS	*F*	P-value
Treatment (between columns)	0.8744	2	0.4372	139500.52	p<0.001
Residual (within columns)	0.000188	6	0.00003133		
Total	0.8746	8			

Table 4.13: Two way ANOVA of *rad51* gene expression in different tissue (blood, gill and muscle tissue) of zebrafish

Source	Type III Sum of Squares	df	Mean Square	F value	Significance
Tissue	2.937	2	1.469	593.522	0.001
Time	1.678	2	0.839	338.958	0.001
Tissue * Time	1.734	4	0.434	175.229	0.001

4.3.2. Gene expression in zebrafish embryo:

A significant difference in gene expression was observed for *sox*2 *sox19a and p53* gene in control and irradiated embryos is represented in Fig. 10, 11 and 12. Statistically significant decrease in the expression of *sox2* gene was observed in the gamma irradiated embryos of zebrafish as compared to the unirradiated control embryos at all the time intervals. The *sox2* gene expression was at 24 hpf (0.52 ±0.024, t-test, $p<0.05$), 72 hpf (0.76 ±0.054, t-test, $p<0.05$) and 120 hpf (0.84 ±0.003, t-test, $p<0.05$). Further, One way ANOVA showed significance different between the time intervals (F= 71.29; *p*< 0.001) summarized in Table 4.14
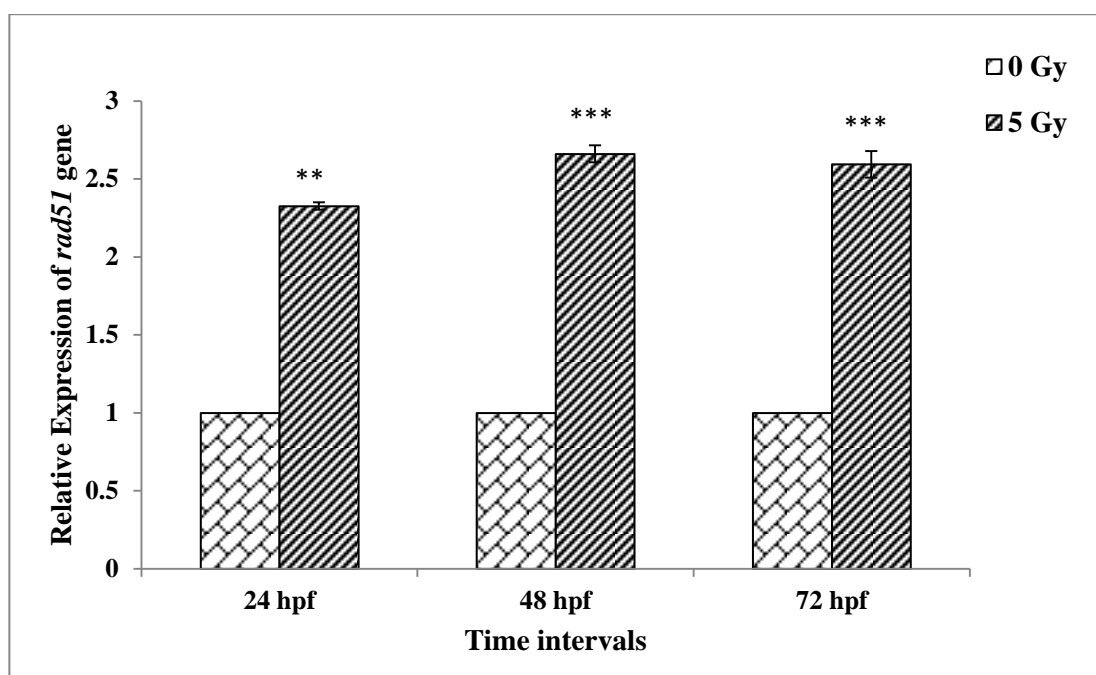
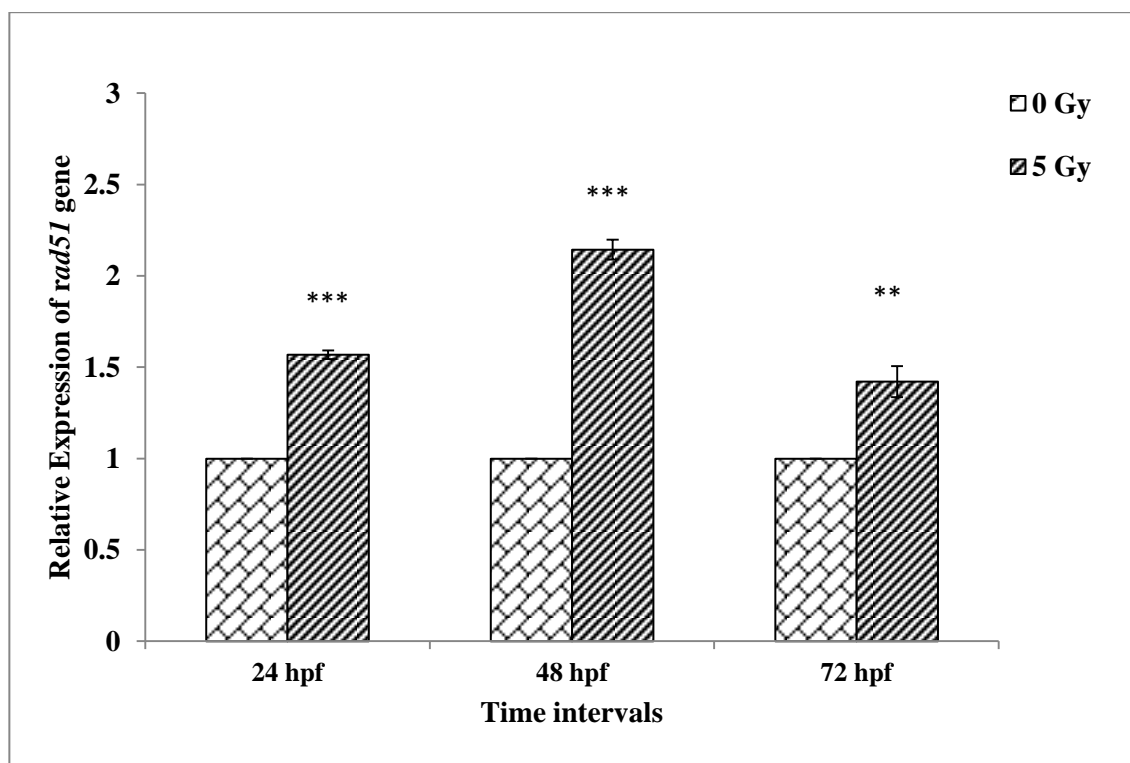**Figure 4.10:** Effect of gamma radiation on *sox2* gene expression in zebrafish embryos. Each value is expressed as the Mean ± S.D. Significant differences between the control and irradiated groups were determined using Student's t-test. Note: ***= P<0.001, ** = P<0.01, * = P<0.05

Table 4.14: One way ANOVA of *sox2* gene expression in zebrafish embryo

ANOVA Table	SS	df	MS	*F*	P-value
Treatment (between columns)	0.1664	2	0.0832	71.29	p<0.001
Residual (within columns)	0.007002	6	0.001167		
Total	0.1734	8			

Further, statistically significant increase in the expression of *sox19a* gene was observed in the gamma irradiated embryos of zebrafish as compared to the unirradiated control embryos. The *sox19a* gene expression was at 24 hpf (2.86 ±0.0284, t-test, p<0.01), 72 hpf (5.75 ±0.006, t-test, p<0.001) and 120 hpf (1.54

±0.003, t-test, p<0.05). Further, One way ANOVA showed significance different between the time intervals (F= 531.9; *p*< 0.001) summarized in Table 4.15.
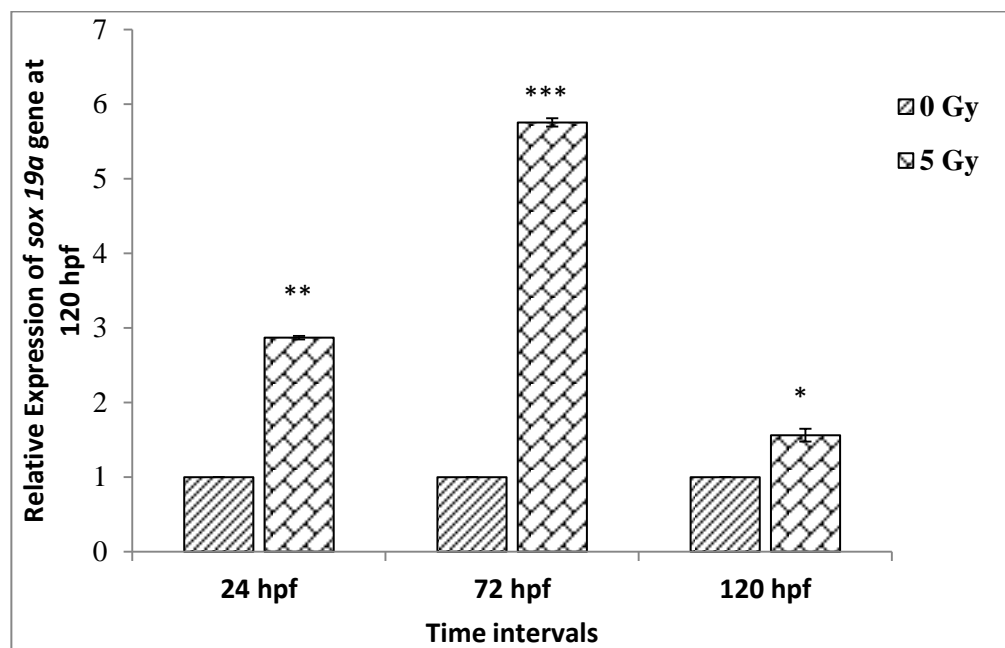
Figure 4.11: Effect of gamma radiation on *sox19a* gene expression in zebrafish embryos. Each value is expressed as the Mean ± S.D. Significant differences between the control and irradiated groups were determined using Student's t-test. Note: ***= P<0.001, ** = P<0.01, * = P<0.05

Table 4.15: One way ANOVA of *sox19a* gene expression in zebrafish embryo

ANOVA Table	SS	df	MS	*F*	P-value
Treatment (between columns)	27.82	2	13.91	531.9	p<0.001
Residual (within columns)	0.1569	6	0.02615		
Total	27.98	8			

Further, statistically significant increase in the expression of *p53* gene were observed in the gamma irradiated embryos of zebrafish as compared to the control embryos. The *p53* gene expression was at 24 hpf (1.35 ±0.023, t-test,

p<0.05), 48 hpf (3.18 ±0.042, t-test, p<0.05) and 72 h (1.20 ±0.066, t-test, p<0.05). Further, One way ANOVA showed significance different between the time intervals (F= 1645; *p*< 0.001) summarized in Table 4.16
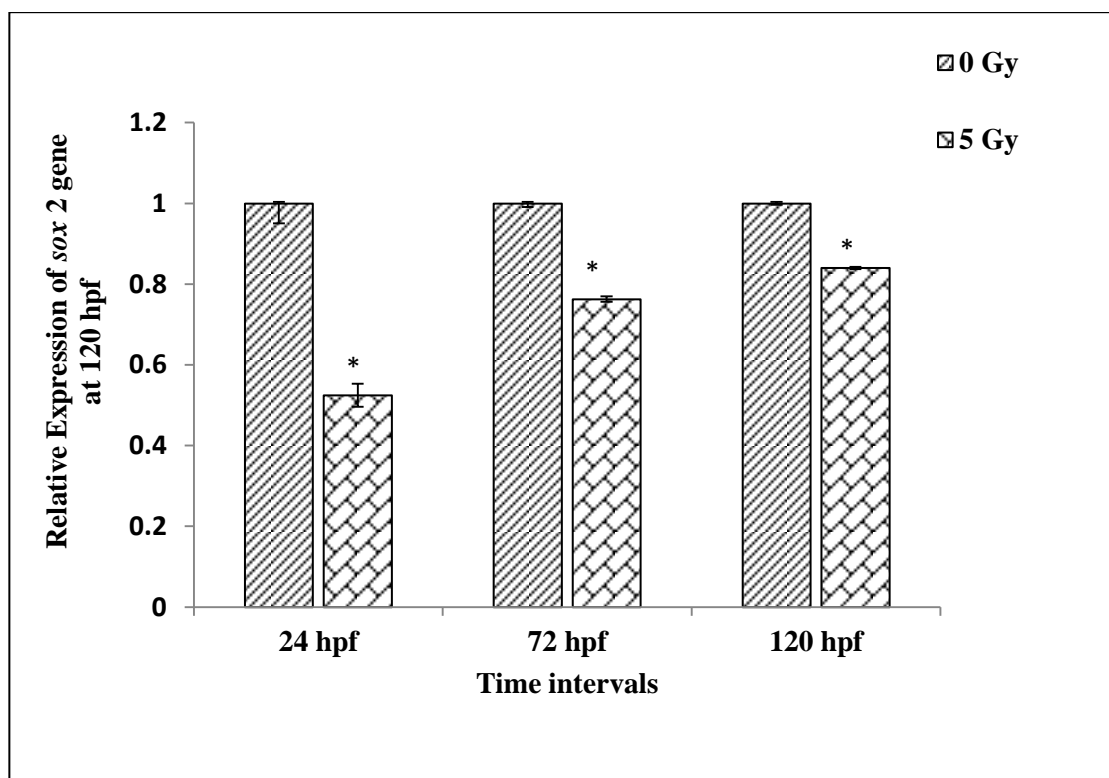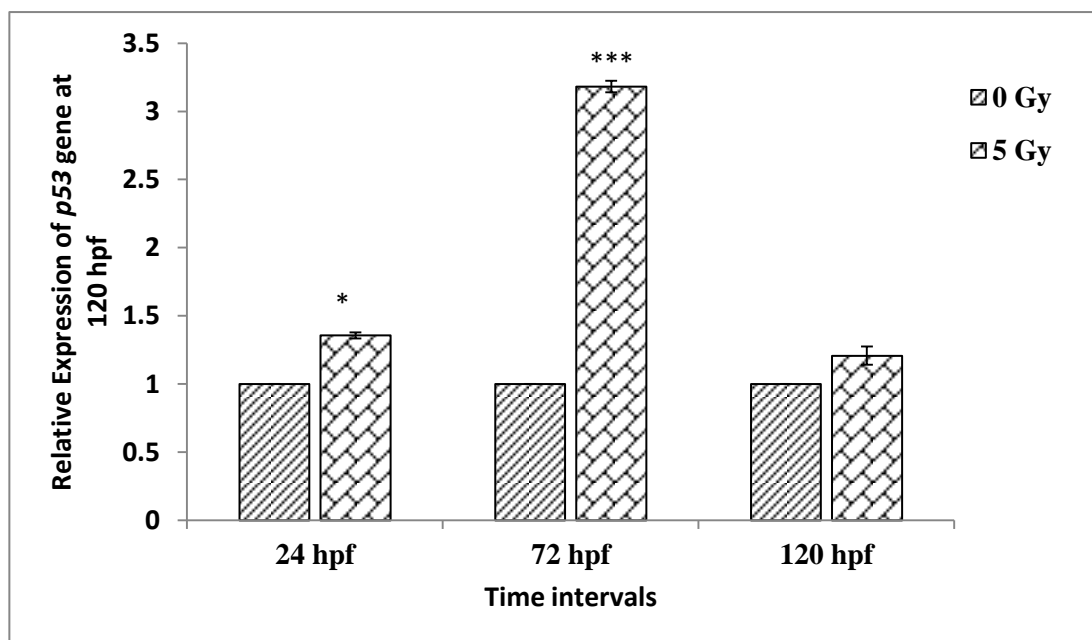

Figure 4.11: Effect of gamma radiation on *p53* gene expression in zebrafish embryos. Each value is expressed as the Mean ± S.D. Significant differences between the control and irradiated groups were determined using Student's t-test. Note: ***= P<0.001, ** = P<0.01, * = P<0.05

Table 4.16: One way ANOVA of *p53* gene expression in zebrafish embryo

ANOVA Table	SS	df	MS	*F*	P-value
Treatment (between columns)	7.292	2	3.646	1645	p<0.001
Residual (within columns)	0.01330	6	0.002216		
Total	7.305	8			

4.4. Discussion:

4.4.1. Zebrafish

When the aquatic organisms are exposed to radiation, the first line of response play major role in the activation of molecules involved in cell cycle arrest, repair processes and apoptosis. These triadic functionalities are imperative to maintain cellular homeostasis. Mechanistic studies about these unique processes in fish after ionizing radiation exposure are limited and will offer new insights about molecular cues on radiation induced DNA damage in aquatic model systems. In the present study, gamma radiation induced a significant up-regulation of target genes *p53, bax* and *rad51* from day 1 to day 3 after irradiation in the zebrafish.

Up-regulation of *p53, bax* and *Rad51* of targets genes in different tissues of zebrafish exposed gamma radiation in present study is on par with Jing et al. (2017). The *p53* is a transcription factor, that play major role in DNA damage inducible molecule that has been reported to influence significant cellular processes, including cell cycle arrest, DNA repair, apoptosis, and senescence. Following irradiation, activation of *p53* promotes cell survival by growth arrest and DNA damage repair (Ding et al., 2013). Significant change in the expression of DNA repair in *Catla catla* expose to gamma radiation. (Anbumani and Mohankumar, 2016). Further, similar observation seen in Atlantic salmon*, (Salmo salar)* exposed to gamma radiation by Song et al. (2014). Lyng et al. (2004) observed similar observation in primary cultures of rainbow trout skin exposed to gamma radiation.

Further, Sandrini et al. (2009a) have also observed activation of the DNA repair systems in the hepatocytes of zebrafish which were exposed to ionizing radiations. Expression level of DNA repair genes could also decide the extent of stability of zebrafish genome in a specific genotoxic stress condition. Several studies in fish have clearly revealed that gene expression can be modulated by exposure to chemical genotoxicants (Gonzales et al., 2005; Sandrini et al., 2009b; Geffroy et al., 2012; Reinardy et al. 2013; Boran snd Terzi, 2017). Heavy metals have also been reported to modulate expression of DNA repair genes. Up-regulation of the *Rad51* gene expression was observed in zebrafish after exposure to methyl mercury (Gonzales et al., 2005). Similarly Copper induced a down-regulation of *p53* and *CDKI* genes as well as an up-regulation of *Gadd45, CyclinG*1 and *Bax* genes in zebrafish cell line ZFL (Sandrini et al., 2009a).

Gamma radiation induced DNA damage in zebrafish is repaired rapidly following induction of various genes involved in DNA repair. Up-regulation of *p53, bax* and *rad51* is consistent with induction of both non-homologous end-joining (NHEJ) and homologous recombination (HR), repair pathways. Induction of gene expression was in agreement with other studies, and within the expected context of genes that are constitutively expressed in cells.

The profile of the gene expression response varied over time for all genes evaluated. Further, we observed significant interaction between different tissue and different time intervals. Timing of induction may be of particular importance for DNA repair-related genes, as the timing of repair genes has been suggested to be critical (*in vitro*) in triggering alternative pathways to repair, such as apoptosis (Kim and Hyun, 2006). If a single time point is selected for analysis of gene

expression without knowledge of the expression profile over time. DNA damage can be repaired by induction of DNA repair pathways. The main two pathways for repair of strand breaks are non-homologous end-joining (NHEJ), mediated by the Ku70-Ku80 protein complex (Schulte-Uentrop et al., 2008; Thacker and Zdzienicka, 2004) and homologous recombination (HR) (Thacker and Zdzienicka, 2004). Initiation of different DNA repair pathways can be dependent on the cell cycle stage (Wu et al., 2008), but NHEJ and HR are also not mutually exclusive and can work simultaneously to repair strand breaks (Rapp and Greulich, 2004; Takata et al., 1998). In spite of extensive information concerning the expression profile of components of the DNA repair system that indicate a general pattern, many contradictory results are found in the literature. Many genes that are induced after DNA damage in some studies are not altered or are repressed in others (Gonzalez et al., 2006; Green et al., 2007; Notch et al., 2007). These discrepancies may be due to the type of cellular injury, the dose ∕ concentration of the used agent, the time after damage that the response was analyzed, the organism ∕ organ ∕ cell differences and other factors. According to Begley and Samson (2004) some factors like the stage of the cell cycle (resting or cycling), the position of the lesion, protein abundance and kinetic ∕ thermodynamic factors that coordinate biochemical events could interfere with the pathway that will be activated after DNA damage. Regarding the complexity of the DNA repair system, some authors consider the elucidation of these mechanisms of action a challenge.

Further, change in the gene expression of DNA repair gene in the present study in zebrafish and the similar observation of Anbumani and Mohankumar,

Molecular cytogenetic studies on the toxic effects of gamma radiation in Zebrafish and Common carp

(2016) in *Catla catla* may indicates the similar passion of repair mechanism in both fish. It may due to they are belongs to same family Cyprinidae.

4.4.2. Zebrafish embryo:

Sox family genes play key roles in maintaining stem cells and progenitors. They are also master regulators of cell fate determination and tissue morphogenesis (Yin, 2017). In the present study we observed significant change in the expression level of *sox*2 and *sox*19a gene has been clearly revealed in gamma irradiated zebrafish embryos. Similar results were observed by Desai et al. (2011) where they studied the effect of chilling and subsequent warming on the expression of developmental genes *sox*2, *sox*3 and *sox*19a in zebrafish. Ma et al., 2017 also observed change the expression of of *sox5*, *sox8* and *sox9* genes in Golden Pompano *Trachinotus ovatus* exposed different water temperature. Okuda et. al. (2010) observed the sox gene proteins control a wide range of developmental regulators in the early embryo and suggest that the sox gene functions are central to coordinating cell fate specification with patterning and morphogenetic processes occurring in the early embryo. Freeman et al. (2014) have also observed a significant decrease in expression of *LIN7B* genes in zebrafish embryos exposed to gamma radiation. Further, up regulation of *p53* genes in embryo in present study is on par with Jing et al. 2017, in which zebrafish embryo is exposed to ^{56}Fe ion radiation. Similar observation also observed by Rhee et al. 2013, where they exposed fish to gamma radiation. In normal healthy cells have very low level of *p53*, but become elevated when cell under go any damage (Haupt et al., 2003). Expression level of DNA repair genes could also decide the extent of stability of zebrafish genome in a specific

genotoxic stress condition. Damages which could not be repaired by their DNA repair system finally might have led to the morphological deformities in the zebrafish larvae (Pereira et al. 2011). Sandrini et al. (2009a) have observed activation of the DNA repair systems in the hepatocytes of zebrafish which were exposed to ionizing radiations. Several studies in fish have clearly revealed that gene expression can be modulated by exposure to chemical genotoxicants (Sandrini et al., 2009b; Geffroy et al., 2012). Significant change in regulation of *sox2* and *sox19a* gene expression in the present study may be due to the sensitivity of the *sox* genes to gamma radiation. This may lead to morphological deformities in the larvae. *Sox9a* gene play essential roles in endochondral bone formation during axial and appendicular skeletogenesis. In this study, p53 was up-regulated at transcriptional level by irradiation at 24 and 72hpf, but no change was found at 120 hpf. The *p53* gene plays major role in regulating the apoptosis by interaction with the mitochondria-dependent pathway and Bcl-2-family proteins (Lee et al., 2013). The role of *p53* played in radiation-induced apoptosis during zebrafish development remains unclear. Since 48-72 hpf is a critical period during the early embryonic development of zebrafish, the up-regulation of p53 at 72 hpf may be crucial in enabling the zebrafish to cope with stress and ensure normal development by inhibiting the induction of "abnormal" apoptosis.

4.5. Summary:

In the present study the expression of *p53*, *bax* and *rad51* was significantly upregulated in gamma irradiated zebrafish. This finding indicates that gamma radiation can induce DNA damage and linked to modulation of the expression of DNA repair-related genes as one of the defense mechanisms against radiation damage. This study provides a better understanding of the

molecular mode of action of defense mechanisms upon gamma radiation in fish. Results also indicate that repair mechanisms were initiated rapidly after DNA damage and that evaluation of gene expression profiles throughout the repair process is essential to establish the time course of these processes. Further, the significant changes in the expression of *sox2 sox19a* and *p53* genes were observed in zebrafish embryo. This data was supported the developmental defects observed in the zebrafish embryo exposed to gamma radiation such as i.e. increased DNA damage, decreased hatching rate, increase in median hatching time, decreased body length, increased mortality rate, increased morphological deformities (Chapter 3). Further, study shows that the potential ecotoxicological threat of gamma radiation on the early developmental stages of zebrafish. Hence, these biomarkers could constitute tools to assess radiation effect.

4.6. References

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Summary of the Thesis

The focus of this thesis was to evaluate genotoxicity, developmental toxicity and expression of DNA repair/developmental genes in gamma irradiated fish and embryos with the aim to adopt an integrated approach to investigate links among genotoxicity and developmental toxicity in model fish species. Further, this study was focused to find out detectable effect at lower doses without impacts at the individual level as well as the NOEL (No Observed Effect Level) dose.

In this study, the $LD_{50/30}$ was found to be 21.6 Gy for common carp and 20.4 Gy for zebrafish. Fishes were irradiated with doses (0.2, 0.4, 0.5, 0.6, 0.8, 1, 2, 4, 6, 8 and 10 Gy) of gamma radiation and their genotoxic effects in different tissues (blood, gill and muscle) were studied employing micronucleus (MN) and comet assays (Alkaline single gel electrophoresis). A significant number of MN were observed in both, the dose and the time dependent studies. The lowest frequency of MN was observed at 24h post treatment, which increased considerably at the later time intervals and reached the maximum by 72 h. The result may indicate that the double-stranded DNA damage might not have been repaired, as indicated by increased micronuclei at later periods. A significant increase in DNA damage was observed as indicated by the increase of % tail DNA damage at all the doses of gamma radiation as compared to controls in both fish species. This showed a dose-dependent increase of genetic damage induced in fishes by gamma radiation. Further, the highest DNA damage was observed at 24 h which gradually decreased with advancement of time i.e. at 48 and 72 h in both species of fishes. This may indicate repair of the damaged DNA and/or loss of heavily damaged cells as the post irradiation time advanced. The present study reveals that gamma radiation induces single strand breaks in DNA as measured by alkaline comet assay.

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Further, the NOEL (No Observed Effect Level) dose was ascertained for various tissues of both the species of fishes. NOEL dose varied from tissue to tissue, species to species as well as from one parameter to the other. In the micronucleus test, NOEL dose observed for erythrocytes and gill cells of common carp were 0.6 Gy and 0.4 Gy, respectively and that of zebrafish was 0.6 Gy for both the types of tissues. In the comet assay, NOEL dose observed in common carp erythrocytes, gill cells and muscle cells were 0.5 Gy, 0.4 Gy and 0.4 Gy respectively whereas it was 0.5 Gy for zebrafish erythrocytes and gill cells and 0.6 Gy for muscle cells. Thus, although both fishes belong to the same family (*Cyprinidae*) they showed different sensitivity to radiation at the tissue specific level. Significant interaction between different dose, time intervals and different tissue in both fishes in the present study shows that both the fishes respond in a similar manner to the effects of gamma radiation.

In addition to genotoxic effects in adult fishes, the irradiated embryos of zebrafish exhibited decreased hatching rate, increased median hatching time, decreased body length, increased mortality rate, increased morphological deformities as well as increased DNA damage. This clearly demonstrated the positive mutagenic effect of gamma radiation on zebrafish embryos. Further, a positive correlation was noted between DNA damage and morphological deformities during embryo development.

In the present study the expression of *p53*, *bax* and *rad51* was significantly upregulated in gamma irradiated zebrafish. Gamma radiation can induce DNA damage and linked to modulation of the expression of DNA repair-related genes as one of the defense mechanisms against radiation damage. This study provides a better understanding of the molecular mode of action of defense mechanisms upon gamma radiation in fish. Results also indicate that repair

mechanisms were initiated rapidly after DNA damage and that evaluation of gene expression profiles throughout the repair process is essential to establish the time course of these processes. Further, the significant changes in the expression of *sox2 sox19a* and *p53* genes were observed in zebrafish embryo. This data was supported by the developmental defects observed in the zebrafish embryo exposed to gamma radiation such as i.e. increased DNA damage, decreased hatching rate, increase in median hatching time, decreased body length, increased mortality rate, increased morphological deformities. Thus, the present study reveals that the DNA damage in the developing embryo could be the possible reasons for the morphological deformities in zebrafish larvae.

In conclusion, both the fishes can be used as model organisms to monitor radiation pollution in the environment. The micronucleus test and comet assay therefore represent essential biomonitoring tools to evaluate gamma radiation exposure and pollution in the environment. The zebrafish embryo can also be used as a sensitive bio-indicator of a genotoxicant within an environmentally realistic range. Therefore, the zebrafish adult as well as embryo can be a model bio-indicator of radiation exposure in aquatic environments, capable of furnishing good measurable responses to such genotoxicants and mutagenic agents.

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