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Using zebrafish as a biological model to study ionizing radiation effects

Tese de Candidatura ao grau de Doutor em Patologia e Genética Molecular submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Em memória de José Lemos,

meu querido Pai

1953 - 2012

Prefácio

O mundo das radiações, na figura da Medicina Nuclear, foi-me apresentado em meados de 2004 (já lá vão mais de 10 anos!). Era eu na altura uma finalista do ensino secundário e andava em busca do meu ofício. Ora deveria ser algo relacionado com a saúde, uma coisa de vanguarda, com muita tecnologia à mistura, uma área em constante inovação, onde pudesse aprender coisas novas todos os dias, talvez investigação científica, mas o melhor seria conciliar tudo isto. Estes meus desejos e dilemas eram muitas vezes partilhados em conversas com a família e o meu Pai, sempre atento e (o maior) conhecedor das minhas ambições, chegou um dia a casa com um "trabalho bem feito" sobre a Medicina Nuclear. "Quando tiveres tempo dá uma olhadela a estes papéis". E pronto. Aquela recolha de informação foi o bastante para me lançar à aventura na área da Medicina Nuclear. Em Setembro de 2004 comecei a frequentar o Curso de Medicina Nuclear da Escola Superior de Tecnologia da Saúde do Porto (ESTSP.IPP) que terminei em Agosto de 2008. Em Novembro do mesmo ano dei início a mais uma aventura: fui convidada para ingressar na equipa docente do mesmo Curso. Daí à entrada no Doutoramento foi um instante.

A escolha do tema da tese de doutoramento foi outra andança. Teria de ser algo relacionado com a Medicina Nuclear, com o mundo das radiações, com as preocupações de um Técnico de Medicina Nuclear, algo que precisasse de ser mais explorado. Estas minhas aspirações eram muitas vezes partilhadas em conversas com os meus colegas de trabalho e o coordenador do Departamento, o Prof. Luís F Metello, sempre atento e (o maior) conhecedor das minhas capacidades, lançou-me o desafio (e o seu desejo antigo) de explorar a utilização do "peixito" *zebrafish* na radiobiologia. Num ápice, o Prof. Luís F. Metello organizou uma reunião com Prof. Doutor Vítor Vasconcelos, a quem apresentou o projeto que tinha em mente. Foi recebido de braços abertos e com imensa curiosidade e interesse, pessoal como institucional, tendo ficado aí acordados os princípios base que nortearam tudo o resto. Poucos dias depois ocorreu nas instalações do Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), a primeira reunião alargada, em que participei e em que me foi apresentado o Prof. Doutor António Paulo Carvalho, que tinha aceite abraçar o projeto e vir a ser o meu Orientador. Posso afirmar que esse foi o dia D desta longa caminhada.

Apesar de lecionar na ESTSP.IPP, e da minha família precisar - nessa fase em particular, por doença grave do meu querido Pai - de uma filha a tempo inteiro e de ter, por isso, limitações de tempo para dedicação exclusiva ao projeto de doutoramento, consegui convencê-los que daria o tudo por tudo e que não sairiam dececionados ao confiarem em mim. Sempre auxiliada por pessoas que nos momentos certos me iluminaram os melhores caminhos (ainda que alguns fossem trilhos bem apertados), chego ao fim desta etapa com a noção que o meu projeto está incompleto, mas com a certeza de ter dado o meu melhor. Superei inúmeros desafios, aprendi técnicas novas, manuseei equipamentos que desconhecia a sua existência, conheci pessoas extraordinárias mas acima de tudo superei-me a mim própria. Sou hoje uma pessoa muito diferente pelo muito que aprendi e partilhei neste período.

Joana Lemos

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(profissionais e pessoais pois nem sempre foi fácil separar estes dois campos)

Este trabalho não seria possível sem o contributo pessoal e/ou profissional de muitas pessoas às quais quero demonstrar a minha (eterna) gratidão.

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Comunicações e Publicações

No cumprimento do disposto no Decreto-Lei nº 230/2009, declara-se que a autora desta tese participou ativamente na conceção e na execução do trabalho experimental que estiveram na origem dos resultados apresentados, bem como na sua interpretação e na redação dos respetivos manuscritos.

No âmbito do trabalho desenvolvido nesta tese, os seguintes trabalhos foram submetidos e aceites para apresentação, sob a forma de poster ou comunicação oral, em várias reuniões científicas nacionais e internacionais. Os resultados foram ainda parcialmente divulgados, por convite, em duas palestras em eventos nacionais.

Posters, comunicações orais e palestras:

Society of Nuclear Medicine and Molecular Imaging - SNMMI 2015 (6-10 Junho 2015, Baltimore, USA)
Poster*: ***Lipid peroxidation and antioxidant responses in zebrafish models after exposure to low doses of ionizing radiation***
*Vencedor do 2ND PLACE TECHNOLOGIST BEST POSTER AWARD

LOWDOSE-PT-2015 (15 e 16 Abril 2015, Lisboa, Portugal)
Comunicação oral: ***Antioxidant and oxidative stress responses in zebrafish brain after exposure to low doses of ionizing radiation***

BIOIMAGING 2014 - 3rd International Symposium in Applied Bioimaging – Capturing Life in a Pixel (16 e 17 Outubro de 2014, Porto, Portugal)
Poster: ***Assessing radiobiological effects of diagnostic level doses of ionizing radiation on zebrafish using comet assay***

Society of Nuclear Medicine and Molecular Imaging - SNMMI 2014 (7-11 Junho 2014, St. Louis, USA)
Poster: ***The acute effect on three-dimensional cellular proliferation rate of low dose irradiation exposures***
Poster: ***Assessing radiobiological effects of diagnostic level doses of ionizing radiation on zebrafish using comet assay***

X Congresso Nacional da Associação Portuguesa de Técnicos de Medicina Nuclear (5 Julho 2014, Lisboa, Portugal)
Palestra: **Radiobiologia aplicada**

26th Annual Congress of the European Association of Nuclear Medicine– EANM'13 (19-23 Outubro 2013, Lyon, França)
Comunicação oral: ***Evaluation of Radiobiological Effects in Three Distinct Biological Models***

Protecção Radiológica na Saúde 2013 (18-20 Setembro 2013, Lisboa, Portugal)
Poster: ***Assessing Radiobiological Effects of Low Doses of Ionizing Radiation on Zebrafish by Two-dimensional Gel Electrophoresis***

Exposição "Radiações: Elas andam aí" organizada pelo Centro Interdisciplinar de Investigação Marinha e Ambiental - CIIMAR (13 Dezembro 2012, Vila de Conde, Portugal)
Palestra: **Efeitos das Radiações Ionizantes nos Tecidos Biológicos**

Proteção 2012 (20-23 Novembro 2012, Lisboa, Portugal)
Comunicação oral: ***Developing advanced biological models to study effects of low doses of ionizing radiation: our experience***

24th Annual Congress of the European Association of Nuclear Medicine – EANM'11 (15-19 Outubro 2011, Birmingham, UK)
Comunicação oral: ***Zebrafish and Nuclear Medicine methods and techniques: an each-day improving partnership***

Faz parte integrante desta tese o seguinte artigo científico original publicado em revista científica indexada:

J. Lemos, T. Neuparth, M. Trigo, P. Costa, D. Vieira, L. Cunha, F. Ponte, P.S. Costa, L.F. Metello, A.P. Carvalho. **"Single Low-Dose Ionizing Radiation Induces Genotoxicity in Adult Zebrafish and its Non-Irradiated Progeny"**. Bull Environ Contam Toxicol 2017 Feb;98(2):190-195 (doi: 10.1007/s00128-016-2006-1)

Resumo

Embora a radiação ionizante esteja presente desde sempre na Natureza (no espaço, na crosta terrestre e até mesmo nos nossos próprios corpos), esta permaneceu ignorada ao longo da história da Humanidade até cerca de um século atrás. Pouco tempo após a descoberta da radiação ionizante tornou-se evidente que a exposição a determinados níveis deste tipo de radiação poderia induzir, quer a curto prazo quer a longo prazo, efeitos negativos nos seres vivos. Os efeitos biológicos da radiação ionizante nos seres vivos resultam essencialmente de danos provocados ao nível do ácido desoxirribonucleico (ADN) - que é considerado o alvo crítico da célula - mas também ao nível das proteínas e dos lípidos.

Estudos epidemiológicos (relacionados com coortes de sobreviventes de bombas atómicas, de diversos acidentes nucleares, de indivíduos sujeitos a irradiação médica, a exposição ocupacional e ambiental), estudos com modelos animais e estudos *in vitro* têm sido importantes fontes de informação para esclarecer os efeitos biológicos das radiações. No entanto, os riscos da exposição a baixas doses de radiação na saúde humana ainda não estão claros. Dado o número crescente de procedimentos médicos que utilizam radiação ionizante, das doses cada vez mais significativas que lhes estão inerentes e da incerteza dos efeitos biológicos reais das baixas doses, mais estudos são fundamentais para iluminar esta problemática.

Apesar do seu limitado uso na radiobiologia, nos últimos anos, o peixe-zebra (*Danio rerio*) tornou-se um modelo preferido na pesquisa biomédica essencialmente devido ao elevado nível de homologia com o genoma humano, complementado por uma grande facilidade de manuseamento/manutenção.

O objetivo de longo prazo desta tese foi explorar o peixe-zebra como um modelo animal para a radiobiologia pela avaliação de importantes biomarcadores e melhorar o conhecimento sobre os mecanismos relacionados com as respostas *in vivo* às baixas doses de radiação ionizante. Para estes fins, vários animais foram expostos a diferentes doses de raios-X (entre 100 a 1000 mGy), tendo sido posteriormente sacrificados em diferentes momentos e recolhidas várias amostras de órgãos. Foi realizado *comet assay* ao sangue dos peixes adultos irradiados assim como à sua descendência (larvas). A resposta antioxidante - por análise da catalase (CAT) - e o dano oxidativo - por análise da peroxidação lipídica (LPO) -

foram investigados no cérebro e no fígado tendo sido ainda estudado o perfil proteómico em tecido muscular dos peixes adultos irradiados.

No que diz respeito às quebras de cadeia do ADN medidas pelo *comet assay*, o nível de danos no ADN dos peixes irradiados foi positivamente dependente da dose no dia 1 pós-irradiação; de seguida, o nível de danos em todos os grupos irradiados tornou-se semelhante ao do grupo controlo, sugerindo a reparação dos referidos danos. O nível de dano no ADN da descendência mostrou-se diretamente correlacionado com a dose de radiação da exposição parental, e refletiu o nível inicial de danos no ADN dos respetivos progenitores.

Respostas diferenciais à radiação por parte de machos e fêmeas foram encontradas com recurso a biomarcadores antioxidantes e de dano oxidativo. Os resultados obtidos sugerem que as fêmeas e os machos apresentam respostas distintas à radiação ao longo do tempo. Os nossos dados apontam para uma possível hiper-radiossensibilidade em doses mais baixas (≤500 mGy).

A análise à expressão das proteínas reconheceu diferenças entre o grupo controlo e os grupos irradiados (27 pontos em 1000 mGy; 22 pontos em 500 mGy; 3 pontos em 100 mGy). A maioria das proteínas expressas diferencialmente foi regulada negativamente nos animais irradiados quando comparado com o grupo de controlo.

Todas estas experiências demonstram que o peixe-zebra deve ser considerado como um modelo pré-clínico válido para a radiobiologia. Com este trabalho provamos que o *comet assay*, a avaliação da atividade da CAT e dos níveis de LPO, a eletroforese bidimensional em gel e a espectrometria de massa pela técnica de *matrix-assisted laser desorption/ ionization - time of flight* (MALDI-TOF) são ferramentas adequadas para estudar os efeitos de baixas doses de raios-X no peixe-zebra. Os parâmetros que estabelecemos neste trabalho devem facilitar futuras investigações em radiobiologia usando o peixe-zebra como um modelo animal no contexto de baixas doses.

Palavras-chave: peixe-zebra; radiação ionizante; raios-X; baixas doses; biomarcadores.

Summary

Although ionizing radiation has always been present in nature (in space, in the earth's crust, in our own bodies), throughout man's history it remained ignored until about one century ago. Very soon after the discovery of ionizing radiation, it became evident that exposure to this kind of radiation could induce short-term and long-term negative effects in living beings. The biological effects of ionizing radiation in a living being result essentially from damage to deoxyribonucleic acid (DNA) - which is the critical target within the cell - but also to proteins and lipids.

Epidemiological studies (from atomic bombs survivors, nuclear accidents, medical irradiation, occupational irradiation and environmental exposure), animal studies and *in vitro* studies have been important sources of information to clarify the biological effects of radiation. However, the risks of exposure to low-dose radiation on human health are still unclear. Given the increasing number of medical procedures using radiation and the uncertainty of the real biological effects of low doses of radiation, more studies are imperative to shed light on this problematic.

Despite its limited use in radiobiology, in recent years, the zebrafish (*Danio rerio*) has become a preferred model in biomedical research essentially due to the level of homology with the human genome, complemented by a quite easy and affordable practical side.

The long term aim of this thesis was to explore the zebrafish as an animal model for radiobiology by assessing important biomarkers and to improve the knowledge about the mechanisms related to the *in vivo* responses to low doses of ionizing radiation. For these purposes, several animals were exposed to different doses (between 100 mGy to 1000 mGy) of X-rays, were sacrificed in different moments and samples of different organs were collected. An experiment with a comet assay protocol was applied to the blood and to the offspring (larvae) of the irradiated fish. The antioxidant response - by the analysis of catalase (CAT) activity - and the oxidative damage - by the analysis of lipid peroxidation (LPO) - were investigated in brain and liver and a study of the proteomic profiles was done in muscle tissue.

Concerning to DNA strand breaks measured by comet assay, the level of DNA damage in irradiated parental fish was positively dose-dependent at day 1 post-irradiation; thereafter the level of damage in all irradiated groups became

similar to that of the control group, indicating DNA repair. The level of DNA damage in the progeny was directly correlated with the radiation dose of parental exposure, and reflected the initial level of DNA damage of respective parents.

Differential responses to dose of males and females were found for antioxidant and oxidative damage biomarkers. Results obtained suggest that females and males present distinct responses over time to radiation. Our data point to a possible hyper-radiosensitivity in lower doses (≤500 mGy).

The analysis of protein expression recognized differences between the control and the irradiated groups (27 spots to 1000 mGy; 22 spots to 500 mGy; 3 spots to 100 mGy). Most differentially expressed proteins were down-regulated in irradiated groups when compared to the control group.

All these experiments demonstrate that zebrafish should be considered as a valid preclinical model to radiobiology. We prove that comet assay, assessment of CAT activity and LPO levels, two-dimensional gel electrophoresis (2DE) and matrix-assisted laser desorption/ ionization - time of flight (MALDI-TOF) mass spectrometry method are adequate tools for study effects of X-rays low-doses in zebrafish. The parameters that we established should facilitate future investigations in radiobiology using zebrafish as an animal model in the context of low-doses.

Keywords: zebrafish; ionizing radiation; X-rays; low doses; biomarkers.

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List of Abbreviations and Acronyms

^{18}F	Fluorine 18
2DE	Two-Dimensional Gel Electrophoresis
ANOVA	Analysis of Variance
APS	Ammonium PerSulfate
ASPA	Animals Scientific Procedures Act
BEIR	Biological Effects of Ionizing Radiation
BOGA	*Biotério de OrGanismos Aquáticos*
BSA	Bovine Serum Albumin
CAT	Catalase
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CIIMAR	*Centro Interdisciplinar de Investigação Marinha e Ambiental*
CNSC	Canadian Nuclear Safety Commission
CT	Computed tomography
CuZn-SOD	Copper zinc-SuperOxide Dismutase
CV	Coefficient of Variation
DNA	DeoxyriboNucleic Acid
dpf	days post-fertilization
DRCA	Database on Radiogenic Cancer in Animals
DSB	Double Strand Breaks
DTT	Dithiothreitol
EDTA	EthyleneDiamineTetraAcetic acid
FDG	FluoroDeoxyGlucose
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
Gy	Gray
H_2O	Water
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
ICRP	International Commission on Radiological Protection
ICRU	International Commission on Radiation Units and Measurements
IEF	IsoElectric Focusing

KCl	Potassium chloride
LD	Lethal Dose
LEGE	Laboratory of Ecotoxicology, Genomics and Evolution
LET	Linear Energy Transfer
LNT	Linear Relationship with No Threshold
LPO	Lipid PerOxidation
MALDI-TOF	Matrix-Assisted Laser Desorption/ Ionization - time of flight
MDA	MalonDiAldehyde
MeSH	Medical Subject Headings
mtDNA	Mitochondrial DeoxyriboNucleic Acid
MU	Monitor Unit
Na_2EDTA	Ethylenediaminetetraacetic acid disodium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCRP	National Council on Radiation Protection and Measurements
O_2	Oxygen
OECD	Organisation for Economic Co-operation and Development
OH•	Hydroxyl
PAGE	PolyAcrylamide Gel Electrophoresis
PDAT	Publication Date
PET	Positron Emission Tomography
RERF	Radiation Effects Research Foundation
ROS	Reactive Oxygen Species
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
Se-GPx	Selenium dependent Glutathione Peroxidase
shRNA	short hairpin RiboNucleic acid
SOD	SuperOxide Dismutase
SSB	Single-Strand Breaks
SSP	Sample Spot Protein
Sv	Sievert
TBARS	ThioBarbituric Acid Reactive Substances
TEMED	TetraMethylEhyleneDiamine

Chapter 1

General Introduction

Although radiation has always been present in nature (in space, in the earth's crust, in our own bodies), throughout man's history it remained unnoticed until about one century ago. In 1895, a scientist by the name of Wilhelm Roentgen, while working with vacuum tubes, discovered by chance what we call X-rays. The following year, Becquerel discovered a similar natural radioactivity in some uranium rocks. In 1898, the spouses Marie and Pierre Curie discovered polonium and radium, two radioactive elements. These were to become important milestones in the development of Industry and Medicine.

Radiation is a form of energy. The term "radiation" is very broad because it includes such things as light, heat and radio waves. There are two types of radiation: ionizing and non-ionizing radiation. In the context of this thesis, "radiation" refers to "ionizing" radiation, that when it passing through matter, it can cause it to become electrically charged → ionized. Ionizing radiation may assume many forms: electromagnetic radiation (such as X-rays or gamma-rays), subatomic particles (such as protons, neutrons) or in the form of alpha and beta particles (Committee to Assess Health Risks from Exposure to Low Levels of Ionizing Radiation 2006).

Very soon after the discovery of radiation, it became evident that exposure to radiation above certain levels could induce short-term and long-term negative effects in living beings.

Radiobiology

Radiobiology is a branch of science concerned with the action of ionizing radiation on biological tissues and living organisms (Podgorsak 2005). It is the result of the combination of two disciplines: radiation physics and biology.

The biological effects caused by radiation depend on radiation quality. Linear Energy Transfer (LET) is the unit of measure used to define the level of radiation quality. The unit usually used for LET is kiloelectron Volt *per* micrometer (keV/μm). The International Commission on Radiation Units and Measurements (ICRU) defines the LET as follows:

"LET of charged particles in a medium is the quotient dE/dl, where dE is the average energy locally imparted to the medium by a charged particle of specified energy in traversing a distance of dl."

Different types of radiation have different levels of LET. Energetic neutrons, protons and heavy charged particles are high LET radiations (>10 keV/μm) while X-rays and gamma rays are considered low LET radiations (<10 keV/μm) and are therefore sparsely ionizing the particles where it passes (Podgorsak 2005).

Radiation exposures are measured in terms of the quantity absorbed dose, which equals the ratio of energy imparted to the mass of the exposed body or organ. The unit of absorbed dose (D) is joules *per* kilogram (J/kg). For convenience, this unit has been given the special name gray (Gy) (Committee to Assess Health Risks from Exposure to Low Levels of Ionizing Radiation 2006; Martin 2006). The notion of "equivalent dose" is also important because different radiations produce different amounts of biological damage even though the deposited energy (i.e. absorbed dose) may be the same. So "equivalent dose" is the absorbed dose corrected by a radiation quality factor that characterizes the damage related with each type of radiation. For X-rays and gamma rays the correction factor is 1. The unit of equivalent dose is Sievert (Sv) (Martin 2006).

In living tissues, the electrical ions produced by ionizing radiation can affect normal biological processes. The biological effects of radiation result essentially from damage to the deoxyribonucleic acid (DNA), which is the most critical target within the cell. Ionizing radiation is a real DNA-damaging agent, producing a variety of lesions in cellular DNA, including over 20 types of base damages, single-strand

breaks (SSB), double-strand breaks (DSB), DNA–DNA and DNA–protein crosslinks (Prise *et al.* 2005). However, there are also other sites in the cell like mitochondria, ribosomes, and lysosomes (Lombardi 2007) that, when damaged, may lead to cell death (Podgorsak 2005).

After the irradiation of a cell, the damage can happen in one of two different ways (Figure 1): direct or indirect. By direct action, the radiation interacts directly with the critical target in the cell. The atoms of the target itself may be ionized or excited, leading to the chain of physical and chemical events that eventually produce the biological damage. On the other hand, by indirect action, the radiation interacts with other molecules and atoms (mainly water, because approximately 70 to 90% of cells is water, consequently it is the most probable target (Lombardi 2007)) within the cell to produce free radicals which are very reactive and can, through diffusion in the cell, damage the critical target within the cell. Indirect action can be modified by chemical sensitizers or radiation protectors (Podgorsak 2005). Most of the observed damages are a combination of direct and indirect actions (Lombardi 2007). Direct action is the dominant process in the interaction of high LET particles with biological material whereas about two thirds of the biological damage by low LET radiations such as X-rays or electrons is due to indirect action (Podgorsak 2005).

The irradiation of a cell will result in one of the subsequent nine believable outcomes (Podgorsak 2005):

1 - No effect;

2 - Division delay: The cell is late from going through division;

3 - Apoptosis: The cell dies before it can divide;

4 - Reproductive failure: The cell dies when attempting the mitosis;

5 - Genomic instability: There is a delay in reproductive failure;

6 - Mutation: The cell survives but has a mutation;

7 - Transformation: The cell survives however the mutation leads to a transformed phenotype and possibly carcinogenesis;

8 - Bystander effects: An irradiated cell may send signals to neighbouring unirradiated cells and induce genetic damage in them;

9 - Adaptive responses: Irradiated cells become more resistant to subsequent irradiation.

Irradiation of any biological system generates a succession of processes that differ enormously by timescale (Figure 1). The physical phase consists of

interactions between charged particles and the atoms of which the tissue is composed. The chemical phase describes the period in which these damaged atoms and molecules interact with other cellular components in rapid chemical reactions. The biological phase includes all subsequent processes. The timescale to consider between the breakage of chemical bonds and the biological effect may be milliseconds to years, depending on the type of damage (Lombardi 2007; MC *et al.* 2009).

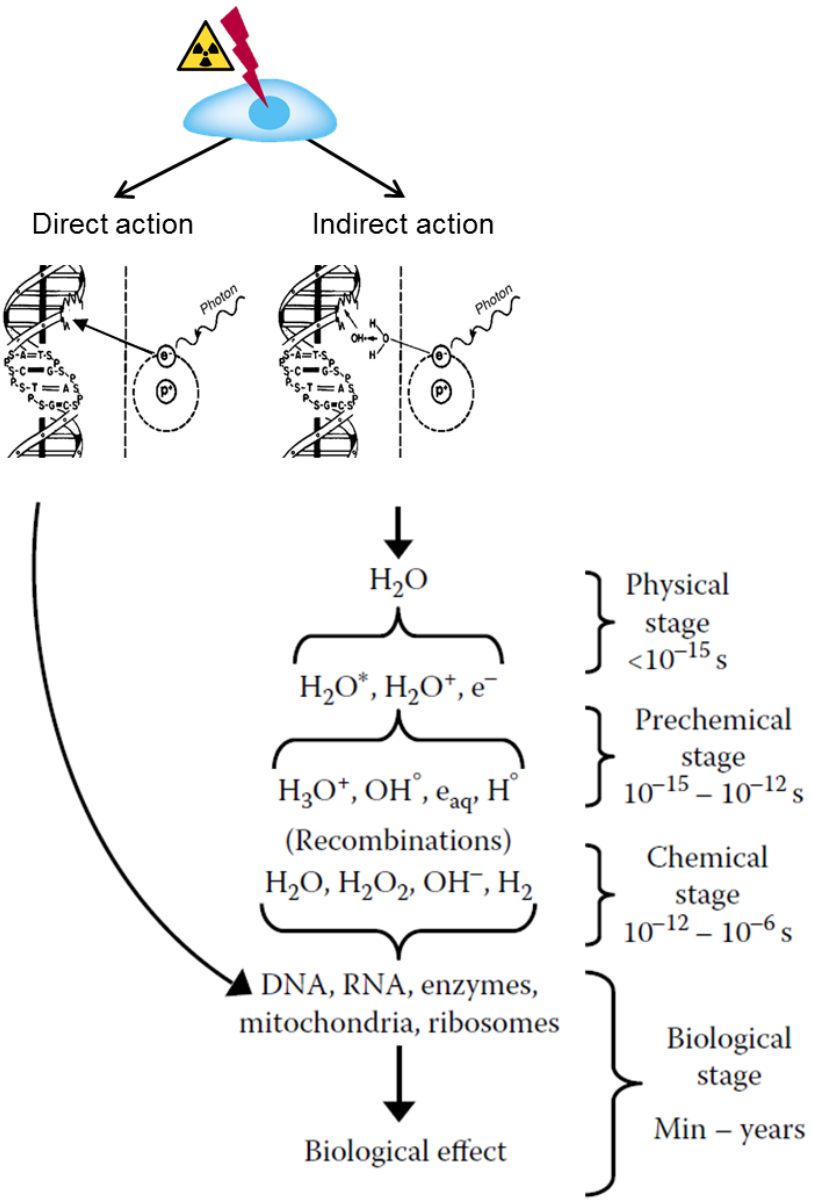

Figure 1 - Timescale of the effects of direct and indirect action of ionizing radiation on biological systems. Adapted from MC *et al.* (2009), Hall *et al.* (2006) and Lombardi *et al.* (2007).

The biological effects of radiation may be classified into two general categories (Table 1): stochastic and deterministic. The definitions of these events by the National Council on Radiation Protection and Measurements (NCRP) are:

- A stochastic effect is present where the probability of occurrence, but not the severity, increases with increased doses. There is no threshold dose for effects that are really stochastic. They may or may not occur in any given exposed individual. They are pure probabilistic phenomena. Examples of these are mutagenesis, carcinogenesis and teratogenesis effects (Podgorsak 2005; Stabin 2008);
- A deterministic effect is present where the severity increases with increasing doses, perhaps after a threshold. Below this threshold, the effects will not be observed, yet above this threshold the magnitude of the effect increases with dose. Examples of these sort of effects are erythema, epilation, fibrosis, lens opacification, blood changes and decrease in sperm count (Podgorsak 2005; Stabin 2008).

Table 1 - Characteristics of stochastic and deterministic effects. Adapted from Podgorsak *et al.* (2005) and Stabin *et al.* (2008).

Stochastic effect	Deterministic effect
↑ dose, ↑probability of occurrence	↑ dose, ↑severity of the effect
Without threshold dose	With threshold dose
Mutagenesis, carcinogenesis and teratogenesis effects	Erythema, epilation, fibrosis, lens opacification, blood changes and decrease in sperm count

When debating deterministic effects, it is important to note that some cells/tissues/organs are more radiosensitive than others. The radiosensitivity of a cell type is proportional to its rate of division and inversely proportional to its degree of specialization. Consequently, fast dividing and unspecialized cells are the most radiosensitive (Bergonie and Tribondeau 1959).

Radiobiological studies

Cohorts of atomic bomb survivors (Preston *et al.* 2007; Little 2009), individuals related with nuclear accidents (Christodouleas *et al.* 2011; Kundiev et al. 2013), with medical radiation (Bhatti *et al.* 2010; Ma *et al.* 2013), with occupational radiation (Cardis *et al.* 2007; Wakeford 2009), as well as with environmental radiation (Hendry *et al.* 2009), together with animal (Duport et al. 2012) and *in vitro* studies (Dhariwala *et al.* 2012; Harder *et al.* 2015) have been very useful helping to clarify what it is already known about the biological effects of radiation.

The biological effects of radiation at high doses and dose rates are quite well documented, and much has been learned by studying the health records from survivors of atomic bombings (Harley 2008). These types of studies involve a large size of the population, both sexes and all ages, wide range of individual doses that are well known and a long follow-up process (Royal 2008). The surviving population of these attacks have been extensively studied over the years. The most important single institution participating in this follow-up effort is the Radiation Effects Research Foundation (RERF), with locations in Hiroshima and Nagasaki. Epidemiologic data from these two tragedies are often associated with the study of high doses however 65% of the survivors of atomic bombings were exposed to 100 mSv or less, that is considered a low dose (Royal 2008).

Many accidents involving ionizing radiation have occurred with industrial and medical sources (UNSCEAR 2000; Bomanji *et al.* 2014). Despite their small number, the accidents associated with nuclear fuel cycle – Chernobyl (1986) and Fukushima (2011) – usually attracted more publicity. Many lessons were learned based on released reports and epidemiologic studies related with these accidents (Williams 2002; Tanaka 2012).

Data from medical radiation studies are often complex to evaluate, essentially due to confounding factors such as the presence of distinct levels and stages of pathologies (Royal 2008).

Some groups of health professionals and nuclear workers are chronically exposed to low doses of ionizing radiation. Since radiation is a weak carcinogen it is difficult to demonstrate a dose-response relationship between cancer and these low doses. The majority of these studies lack statistical power to detect increased

cancer risks regarding doses below 100 mGy (Duport *et al.* 2012; Pernot *et al.* 2012). The cooperation of many companies/countries would be necessary to collect data during many years, in order to have sufficient power to detect these small expected effects (Royal 2008).

Environmental studies should be analysed with caution because ecologic studies based on average population doses and average cancer rates often are associated with significant biases (Lubin 1998).

Despite the fact that epidemiological studies in humans represent an important source of information on health risks associated with exposure to ionizing radiation (Martin *et al.* 2009), they may have limitations like the confounding of unmeasured covariates, such as socioeconomic factors (Mao *et al.* 2001), exposure misclassification and selection and/or recall bias (Wall *et al.* 2006). Since epidemiological studies may not be sensitive enough to detect weak biological effects and to give information of their biological mechanisms, there is a need for a new approach to study the biological effects of radiation on cells, tissues and organisms at all dose ranges, mainly in the low-dose ranges.

In vitro studies are crucial for understanding the biological mechanisms underlying the adverse health effects of radiation especially in the low dose range. However biological endpoints observed in cells may not be directly indicative of radiation-induced carcinogenesis in living organisms (Wall *et al.* 2006).

Studies in experimental animals are important sources of information about biological effects of radiation. Even though the results of animal experiments cannot be directly applicable to humans, these studies can improve for example the knowledge about the relationship between dose and cancer risk for a wide range of doses, as well as the cellular mechanisms by which cancer may develop. The conscientious use of animals in a laboratory environment has many advantages. Some to be considered are the possibilities of using a homogeneous population with relatively little biological variability, the elimination of confounding factors by the randomization at the experimental design stage (Gart *et al.* 1986) and the control of the conditions with reliable estimates of radiation doses (UNSCEAR 2000).

Dose-response model

The relation between dose and its effect is commonly analysed as a plot. A plot comparing an observed biological effect to dose is called a dose-response curve.

There are many types of dose-response relationship (Figure 2) but in general as dose increases so does the effect (Podgorsak 2005).

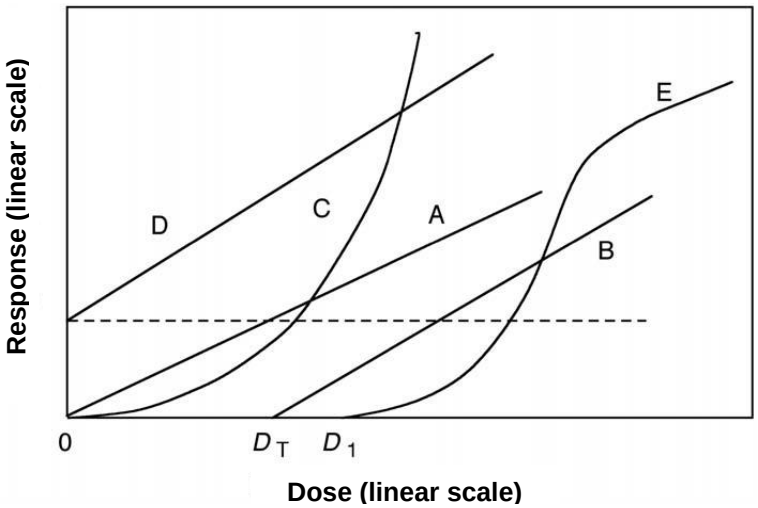

Figure 2 - Dose-response curves: A - a linear relationship with no threshold; B - a linear relationship with a threshold; C - a linear quadratic relationship with no threshold; D - linear relationship; E - a sigmoid relationship with a threshold. Adapted from Podgorsak *et al.* (2005).

The dose-response curves may or may not have a threshold wherein a threshold dose is the largest dose for a particular effect studied, below which no effect will be observed. Typical dose-response curves for cancer induction (curves A, B, C and D) and for tissue response (curve E) are represented in Figure 2, according to Podgorsak (2005), Lombardi (2007) and Little (2003):

- Curve A represents a linear relationship with no threshold (LNT). The risk of effects is linear with dose at all levels. The equation is:

$$y = \alpha D \quad (1)$$

  where y = risk, α = proportionality constant (slope), and D = dose.

- Curve B represents a linear relationship with threshold D_T. There is no risk at low doses. The risk increases linearly with dose above a threshold. The equation is:

$$y = \alpha D + b \quad (2)$$

where α = proportionality constant (slope), D = dose, and

b = Y-intercept at D = 0.

- Curve C represents a linear quadratic relationship with no threshold. The risk of late effects is low at low doses and proportional to the square of the dose at medium and at high doses. The equation is:

$$y = \alpha D + \beta D^2 \quad (3)$$

where α, β = components of cellular change, and D = dose.

- Curve D represents a linear relationship with no threshold (the area below the dashed line represents the natural incidence of the effect).
- Curve E represents a sigmoid relationship with threshold D_1, as is common for deterministic effects in tissues, for example tumour control or treatment morbidity.

The LNT (curve A) is a risk model used internationally by most health agencies and nuclear regulators, including the International Commission on Radiological Protection (ICRP), the U.S. National Research Council of the National Academies and the Canadian Nuclear Safety Commission (CNSC), to establish dose limits for workers and members of the public. As mentioned, epidemiological studies of atomic bomb survivors, environmental, occupational and medical exposures have recognized clear links between radiation exposure and manifestation of cancer (Martin *et al.* 2009). In fact, the cause-effect relationship between radiation exposure and cancer is very complex (and perhaps erroneous) because a radiation-induced cancer is indistinguishable from a “spontaneous” cancer (Stabin 2008). Despite the obvious importance that these studies bring to the understanding of biological effects of radiation, they have not been able to detect in humans a significant increase of the incidence of cancer for doses below 100 mSv because:

- There exists a difficulty of identifying if the effects are caused by the natural incidence of disease or have been caused by radiation;
- Low statistical power of existing studies;
- For the respective threshold, the dose is simply not sufficient to cause cancer.

For these reasons, the only method for estimating the possible risks of such low doses is extrapolation from carcinogenic effects observed at higher doses (Tubiana 2005; Breckow 2006).

By assuming a linear relationship between dose and cancer induction at all levels, the LNT model can overestimate the effects of low doses (Tubiana 2005). This overestimation may have the inconvenience of increasing the cost of radioprotection for industries and hospitals and further increase the fear of the population who may refuse to perform medical exams involving ionizing radiation (Charles 2006; Tubiana *et al.* 2006). However, taking into account the existing uncertainties of low-dose radiation, an alternative model would be impractical for the purposes of radiation protection (Royal 2008).

Despite extensive recognition of the LNT model for radiation protection purposes, several alternative theories (UNSCEAR 2013), represented in the Figure 3, have been proposed to describe the relationship between radiation exposure and cancer risk.

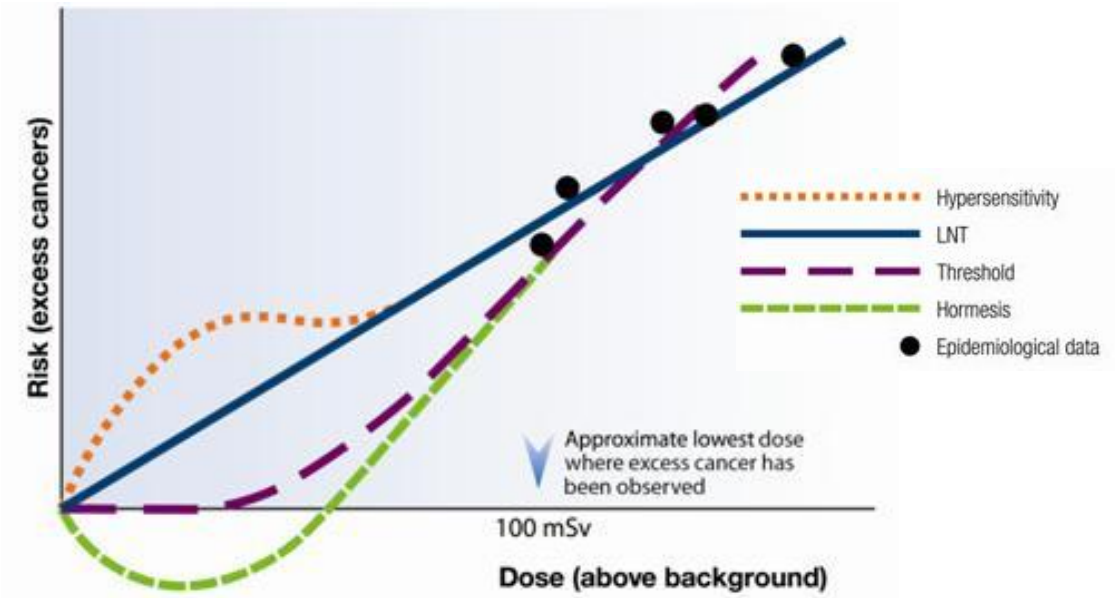

Figure 3 - Alternative dose-response curves: the Hypersensitivity model suggests a greater risk at lower doses; the Threshold model implies that below a certain dose, there is no risk; the Hormesis model suggests that low radiation doses may even be protective and beneficial. Adapted from (Canadian Nuclear Safety Commission 2013).

Many research groups have proposed these alternate theories based on cellular and sub-cellular responses to radiation at low doses (<100 mSv) that do not follow the LNT model. The LNT model is based on the (initial) idea that any DNA lesion has the same probability of promoting cancer regardless the number of

lesions in the same cell and the neighbouring cells (Tubiana *et al.* 2006; Martin *et al.* 2009). During the past two decades, several works have demonstrated the role of interactions between the initiated cell and the surrounding cells. These works caused a change focusing away from a totally DNA-centric approach to include models that invoke complex signalling pathways in cells and between cells within tissues (Barcellos-Hoff 2005; Prise *et al.* 2005; Martin *et al.* 2009).

Two divergent concepts regarding DNA repair have appeared (Royal 2008). Some authors defended that a small amount of radiation could stimulate DNA repair mechanisms (the mechanism of adaptive response) and so result in a beneficial and protective effect, giving rise to the hormesis model (green line in Figure 3) (Dimova *et al.* 2008). This model gives reasons to state that the LNT hypothesis exaggerates risks at low doses and that trying to reduce exposures to background levels can be a waste of time and resources (Stabin 2008).

In contrast, hypersensitivity (orange line in Figure 3) was observed where damage occurs not only to the cell that was exposed to radiation but also to surrounding cells - bystander effect. This model suggests that radiation is more damaging than previously thought. The mechanisms related with the radiation induced bystander are not fully understood but gap-junction intercellular communication (Mancuso *et al.* 2008), the secretion of soluble factors from irradiated cells to the bystander cells (Khan *et al.*) and the reactive oxygen species generated were found to play important roles to this effect.

The bystander effect is usually induced by the more-damaging like α-particles, whereas the adaptive response is typically induced by gamma-rays (Bonner 2003) and there is evidence to support both views.

Despite evidences that supports other models, reports by the International Commission on Radiological Protection (ICRP 99, 2006) and the U.S. National Research Council of the National Academies (BEIR VII, 2006) state that the LNT model still provides the best overall fit for radiation protection purposes.

Why the studied dose range?

The doses of radiation used in this study were 100 mGy, 500 mGy and 1000 mGy. These doses were selected taking into account (1) the application of ionizing radiation in medicine, at diagnostic and therapeutic level, (2) the uncertainties related to cellular and sub-cellular responses to radiation at low doses, and (3) a gap of data on the radiobiological effects of doses between 500 mGy and 1000 mGy in laboratory animals.

Low-dose radiations have become omnipresent in the human environment. A relationship between chronic low-dose radiation and carcinogenesis has been assumed, and injuries in the genetic material (Kovalchuk *et al.* 2004), cellular responses (Russo *et al.* 2012), and developmental dysfunctions (Miyachi *et al.* 2003) caused by low-dose radiations have been reported, yet the risks and long-term impacts of intermittent or continuous exposure to low-dose radiation on human health continue to be unclear (Kovalchuk *et al.* 2004).

According to a NCRP report, Americans were exposed to more than seven times as much ionizing radiation from diagnostic medical procedures in 2006 than they were in the early 1980s (Schauer and Linton 2009). The principal factor responsible for this increase was the appearance and raise of computed tomography (CT) examinations that result in higher organ doses of radiation than conventional single-film X-rays. This is because CT scanners rotate around the body, causing several cross-sectional X-rays expositions. Depending on the machine settings, for CT scanning, organs in the beam can receive doses of about 10 –100 mGy, but that are usually in the range of 15–30 mGy per single CT sequence with an average of two to three CT scans per study (resulting in a dose in the range of 30 to 90 mSv) (Brenner and Hall 2007; Mettler *et al.* 2008).

For this diagnostic field (Table 2) an acute exposure is usually less than 100 mSv nevertheless this value can be exceeded for some whole body CT, positron emission tomography- computed tomography (PET-CT) and fluoroscopy (Brenner 2014).

It is important to note that if the number of diagnostic radiation procedures increases, the exposure of radiation workers (e.g.: nuclear medicine technologists, radiologists) will also increase (Prasad 2012).

Table 2 - Summary of estimated effective dose from typical diagnostic radiation procedures. Adapted from Mettler *et al.* (2008).

Type of Examination	**Average Effective Dose** (mSv)
Standard radiography	0.01 – 1.5
Computed tomography	2.0 – 16.0
Conventional Nuclear medicine	0.2 – 40.7
Positronic Nuclear Medicine (^{18}F-FDG)	14.1
Interventional procedure	5.0 – 70.0

^{18}F- fluorine 18, FDG-fluorodeoxyglucose

At the therapeutic level, an acute exposure below 1.0 Gy has been used in low-dose radiotherapy. The anti-inflammatory efficiency of low-dose radiotherapy was confirmed in several experimental *in vitro* and *in vivo* models. This method uses single fractions below 1.0 Gy and a total dose below 12 Gy to exert anti-inflammatory and analgesic effects on several inflammatory diseases and painful degenerative disorders (Rodel *et al.* 2007).

As already mentioned, several recently recognized responses to low doses of ionizing radiation have been classified as so-called non-targeted responses (Ward 2002). These responses include signalling pathways that are not dependent on DNA damage, bystander responses (Lorimore *et al.* 2003), adaptive responses (Wolff 1998), low-dose hypersensitivity (Joiner *et al.* 2001) , genomic instability (Lorimore *et al.* 2003) and the inverse dose-rate effect (Miller *et al.* 1993).

Related to this issue of low doses, a comprehensive database of animal carcinogenesis experiments was assembled involving exposure to different types of ionizing gradation in this range of dose (i.e. below to 1 Gy). The Database on Radiogenic Cancer in Animals (DRCA) includes all accessible data (after year 2000) related with the radioinduction of cancer in laboratory animals, wherein

approximately 55% of the doses from X-rays were below 500 mGy. It is identified that there is a gap between 0.5 and 1 Gy (Duport *et al.* 2012).

Given the importance of this latest fact, the increasing number of medical procedures using radiation, the increasing dose that are inherent in them and the uncertainty of the real biological effects of low doses of radiation, more studies are imperative to shed light on this problematic.

Biomarkers

A biomarker can be defined as ‘‘any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease. Biomarkers can be classified into markers of exposure, effect and susceptibility’’ (WHO 2001).

One of the bigger challenges in epidemiological studies is the exploration for biomarkers that can permit (Pernot *et al.* 2012):

- The estimation or validation of received dose, improving the validity of a correlation between exposure and biological responses;
- The investigation of individual susceptibility, allowing an individualized therapy.

  Actually, in radiotherapy approaches, the total dose provided is adjusted to the most sensitive individuals resulting in no more than 5% of the patients who will suffer severe adverse healthy tissue effects (Kuhnt *et al.* 1998; Alsbeih *et al.* 2003). Therefore, the majority of the patients will receive a suboptimal dose which may result in cancer recurrence (Dahl *et al.* 1994; Kuhnt *et al.* 1998). So, if radiotherapy could be individualized based on radiation sensitivity, a high number of patients would be cured and the most severe adverse reactions could be avoided (Dahl *et al.* 1994);
- The early detection of a radiation induced health effect.

Pernot *et al.* (2012) classified the potential biomarkers for ionizing radiation exposure as (a) cytogenetic biomarkers, (b) biomarkers related to nucleotide pool damage and DNA damage, (c) biomarkers related to germline inherited mutations and variants, (d) biomarkers related to induced mutations, (e) biomarkers related to transcriptional and translational changes, (f) biomarkers related to epigenomic modifications, and (g) other biomarkers, including biophysical markers of exposure (Figure 4).

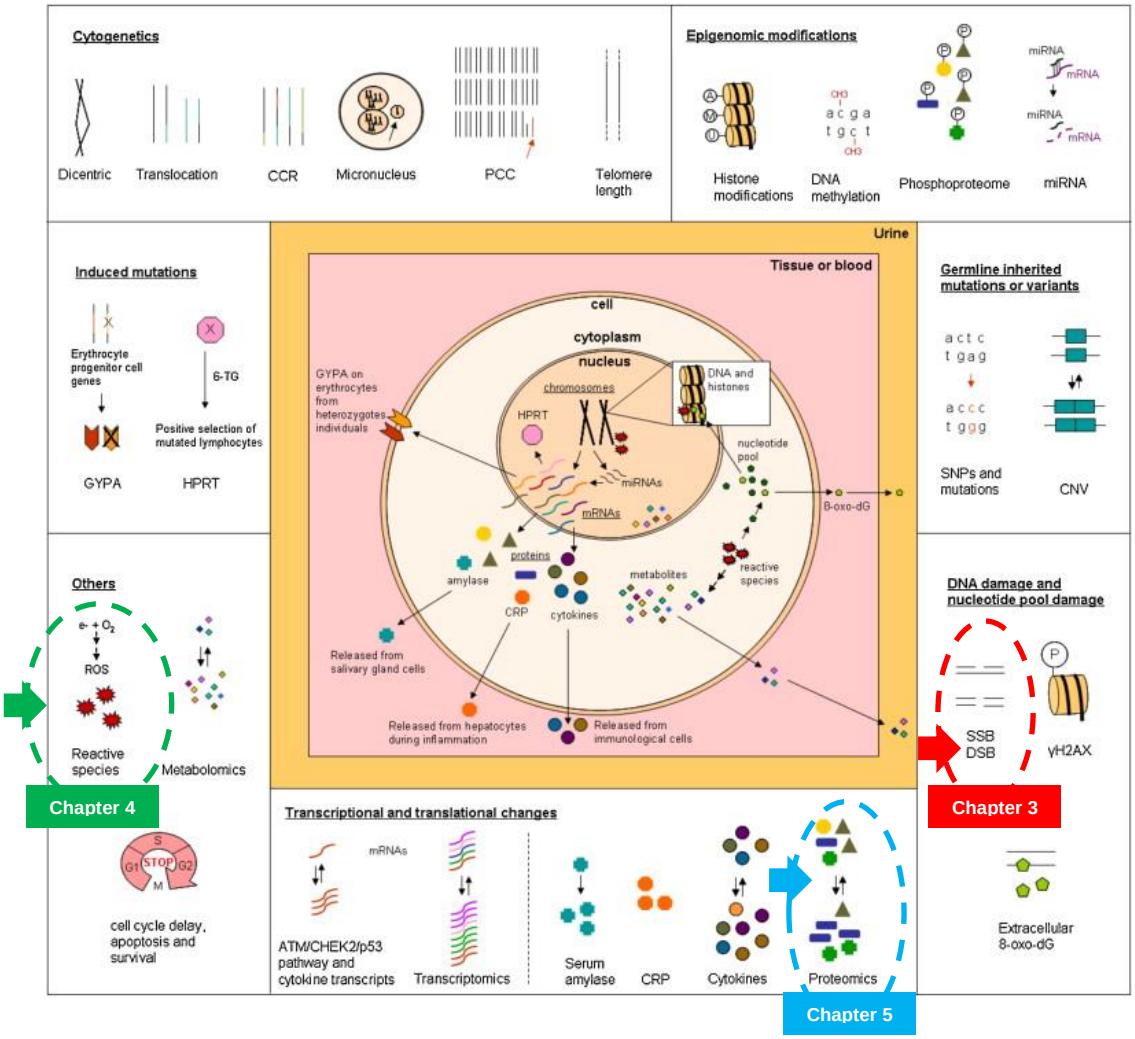

Figure 4 - Overview of the biomarkers of ionizing radiation. Adapted from Pernot *et al.* (2012).

This thesis focuses on the study of three biomarkers: SSB and DSB assessed by comet assay (see Chapter 3), reactive species estimated by antioxidant enzymes activity (see Chapter 4) and proteomic analysis (see Chapter 5). Consequently, a brief analysis of these three biomarkers is presented below.

SSB and DSB assessed by comet assay

The biological effects of radiation in a human or any other living being result essentially from damage to the DNA, which is the most critical target within the cell (Lombardi 2007). Ionizing radiation can induce a multiplicity of DNA damage. For instance, the exposure of mammalian cells to 1 Gy of gamma radiation can result in approximately 1000 SSB, 500 damaged bases, 40 DSB and 150 DNA-protein cross-links (Goodhead 1994; Charles 2001). For low-LET radiation, like X-rays and

gamma radiation, DNA damages are mostly isolated, SSB (Pouget and Mather 2001). The extent of DNA damage, namely strand breaks, can be quantified using comet assay (Pernot *et al.* 2012).

The comet assay - single-cell gel electrophoresis - is a simple, sensitive, economic, rapid and quantitative technique for measuring DNA damage in individual eukaryotic cells (Collins 2004; Olive and Banath 2006; Dhawan *et al.* 2009; Liao *et al.* 2009; Nandhakumar *et al.* 2011). This technique has been a widely used tool for research since 1984 (Ostling and Johanson 1984), particularly in toxicology (Rocco *et al.* 2012), ecological monitoring (Kammann *et al.* 2004), human biomonitoring (Garaj-Vrhovac *et al.* 2002), nutritional studies (Jenkinson *et al.* 1999) and of course in radiobiology (Mikloš *et al.* 2009; Nair and Nair 2013).

Briefly, first the cells are fixed in agarose on a microscope slide and then they are lysed to form nucleoids (protein-depleted nuclei) in which the DNA is still preserved as supercoiled loops connected to the nuclear matrix. Thus, one strand break will relax the supercoiling in the loop in which it occurs, freeing that loop to extend under the electrophoretic field. Electrophoresis results in structures resembling comets when observed by fluorescence microscopy (Collins 2004). Determination of the relative amount of DNA that migrates provides a simple way to measure the number of DNA breaks in an individual cell.

This method has multiple advantages (Gedik *et al.* 1992; McKelvey-Martin *et al.* 1993; Tice *et al.* 2000; Lee and Steinert 2003; Olive and Banath 2006; Kumaravel *et al.* 2009; Erkekoglu 2012) such as the fact that only about a thousand cells are required (which is a very important aspect in our case due to the small size of our animal model), the collection of data at the level of the individual cell (allowing more robust statistical analyses), the results can be obtained in a single day and it has a high sensitivity (as few as 0.1 DNA breaks per 10^9 dalton are detected).

Nevertheless we can point out some disadvantages: single cell data (which may be rate limiting), small cell sample (leading to sample bias), technical variability (quality of protocol and experimental performance is very important especially during electrophoresis), subjective interpretation (Dhawan *et al.* 2009), it is not particularly good for distinguishing apoptosis from necrosis (O'Callaghan *et al.* 2001), it is not able to detect small DNA fragments (<50kb) neither mitochondrial DNA damage (Erkekoglu 2012).

Antioxidant and oxidative stress biomarkers

As already mentioned, by indirect action the radiation interacts with other molecules and atoms (mainly water) within the cell to produce free radicals - atom, molecule, or ion that contains one or more unpaired electrons (Evans and Halliwell 1999) - which are very reactive and can, through diffusion in the cell, damage the critical target within the cell (Azzam *et al.* 2012). After irradiation, reactive oxygen species (ROS, e.g., oxygen ions, free radicals, and peroxide) are produced in cells over several minutes or hours (Riley 1994) where 1 mGy of low-LET radiation causes on average 150 ROS per cell (Feinendegen 2005).

The excess of free radicals can induce oxidative stress (Roots and Okada 1975) that is an imbalance between the creation of free radicals and their removal by specific antioxidants (Riley 1994). Oxidative stress has been associated with many diseases (Thanan *et al.* 2014) like cancers (Wiseman and Halliwell 1996), Parkinson's disease (Tsang and Chung 2009), Alzheimer disease (Galasko and Montine 2010) and cardiovascular disorders (Suzuki *et al.* 2006). Therefore the organism must have protective mechanisms for the removal of excess free radicals (Noctor and Foyer 1998).

Exploration of the oxidative responses in different *in vivo* models has found that cells, tissues and organs contain diverse antioxidant defence systems that form the basis for the differential susceptibility to oxidative environments (Si *et al.* 2013). Similar to what occurs with humans, aquatic organisms have developed a physiological antioxidant system to react to damage from oxidative stress, which implicates antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR), and free-radical traps, such the glutathione molecule and vitamins E and C (Figure 5). These components have an important role against excess free radical and reactive species induced damage (Riley 1994; Si *et al.* 2013; Hou *et al.* 2015).

CAT mostly located in peroxisomes (Evans and Halliwell 1999), is an antioxidant enzyme that catalyses the decomposition of hydrogen peroxide (H_2O_2) into water (H_2O) and (oxygen) O_2. It protects the cell against oxidative stress induced by H_2O_2 or consequently formed hydroxyl radical ($OH\bullet$) (Droge 2002; Weydert and Cullen 2010; El-Bahr 2013).

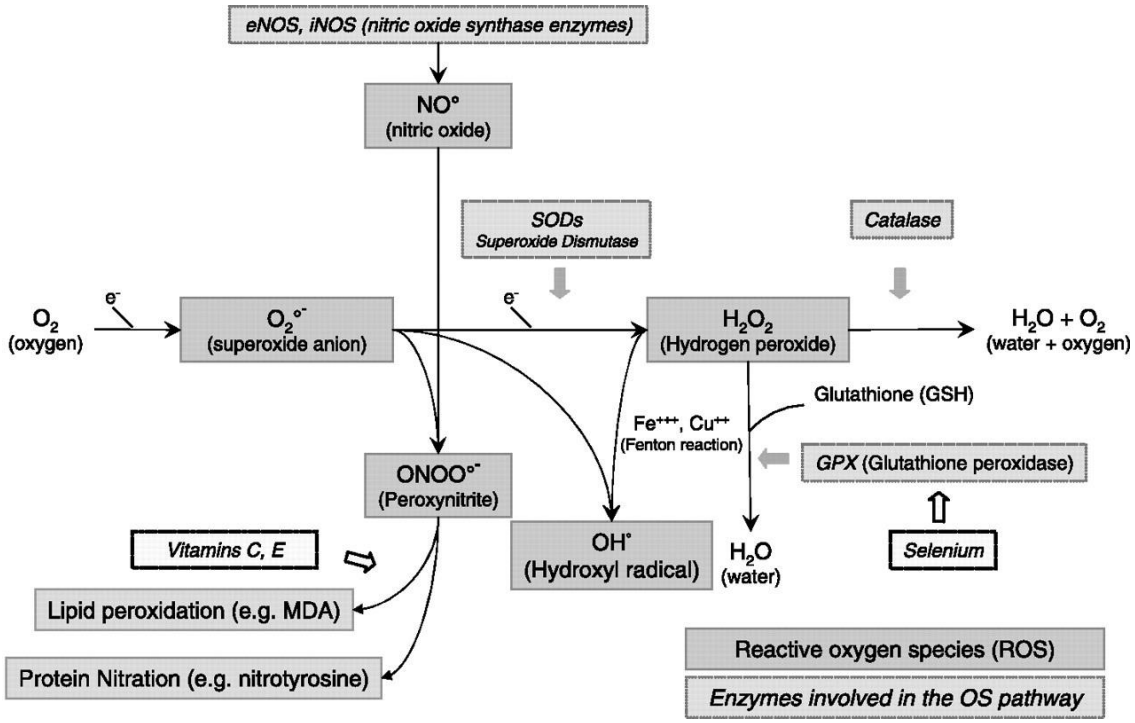

Figure 5 - Pathways of reactive oxygen species formation and clearance. Adapted from Djamali *et al.* (2007).

Lipid peroxidation (LPO) - oxidation of polyunsaturated fatty acids - is a very important consequence of oxidative stress due to extensive production of ROS and/or reduced protective capacity of antioxidant system (e.g. CAT) (Boveris 2008). Malondialdehyde (MDA) is an indicator of oxidative damage that determines the level of lipid peroxidation (Benderitter *et al.* 2003).

Proteomic analysis

Since proteins are the major catalytic and structural components within all living systems, these biomolecules can be an important source of information to understand cellular function and response to any perturbation, such as radiation exposure (Leszczynski 2013).

Meanwhile the tissues will respond to radiation by altering the level of protein expression, it is reasonable to consider that protein expression profiling can be used to find radiation-associated protein biomarkers directly in those tissues or by blood, urine or saliva analysis. Protein radiobiomarkers deployment is still an enormous challenge because of the time- and dose-dependent variation of protein expression (Pernot *et al.* 2012). Despite the high potential of the proteomic for the radiobiology, few studies examined the proteome in cells exposed to ionizing radiation

(Leszczynski 2014). These research groups used animal and cellular models to study potential biomarkers that may then be tested in molecular epidemiological studies (Tapio *et al.* 2005; Guipaud *et al.* 2007; Azimzadeh *et al.* 2011; Pluder *et al.* 2011). Marchetti *et al.* (2006) piloted a literature review about candidate protein biomarkers for individual radiation biodosimetry of exposure to ionizing radiation.

Proteomic methods can be classified into two major categories (Baggerman *et al.* 2005): gel-based (one or two-dimensional gel electrophoresis) and gel-free (liquid chromatography) methods. Gel based proteomics, largely centred on two-dimensional electrophoresis, is an excellent technique for simultaneously visualizing and quantifying up to 2000 protein spots within the same gel (Chevalier 2010). After analysing the gel, the spots predicted to contain biomarkers can be identified using mass spectrometry.

In the radiobiology context, the proteomic approach is an excellent tool mainly for the discovery of new radiobiomarkers but it was used to study bystander effects too. Smith *et al.* (2007) analysed bystander signals emitted by irradiated rainbow trout using a gel-based proteomics approach. The protein profiles of the gills of irradiated fish were different from those of the non-irradiated or the bystander fish. After protein identification, they concluded that the proteins affected in the bystander fish were involved in defence and restorative pathways, suggesting a pre-emptive induction of protective functions.

Why zebrafish?

Studies on biological effects of ionizing radiation using animal models are of extreme importance due to limitations in extrapolating results from *in vitro* cell cultures to *in vivo* processes.

Models are used to represent complex problems in simplified forms – physics, chemistry and biology all make good use of models. In life sciences, the concept of modelling can extend further to include experimental procedures and nonhuman subjects (Levin and Cerutti 2009).

In recent years, the zebrafish (*Danio rerio*) has become a preferred model in biomedical research because of the full knowledge of its genome and the homology it shares with the human genome. The zebrafish (Figure 6) has grown into one of the most important vertebrate model organisms used in genetics, biology, neurophysiology (Vascotto *et al.* 1997; Grunwald and Eisen 2002; Rubinstein 2003; Amsterdam and Hopkins 2006) and its potential in radiobiology has not been ignored (Miyachi *et al.* 2003; McAleer *et al.* 2005; Geiger *et al.* 2006; Hwang *et al.* 2007).

Figure 6 - Adult zebrafish (In: http://www.arkive.org/zebra-danio/danio-rerio/image-G112758.html).

It is a small tropical freshwater fish which inhabits rivers of South Asia, mainly northern India as well as northern Pakistan, Nepal and Bhutan (Dahm *et al.* 2006). The name *Danio* derives from the Bengali name "*dhani*" that means "*of the rice field*" (Talwar and Jhingran 1991).

It has an amount of qualities that make it particularly attractive to experimental manipulation. There is no other vertebrate model organism that offers the same combination of characteristics. The zebrafish combine the advantages of the adults' small size with its large offspring of transparent and easily accessible embryos.

Adults' small size

Zebrafish rarely exceeds 40 mm standard length (from the tip of the snout to the origin of the caudal fin) (Barman 1991). This greatly reduces housing space and husbandry costs. Maintenance costs of zebrafish are less than 1/1000th of the cost of mice (Goldsmith and Solari 2003). There are several companies specializing in zebrafish aquaria capable of supporting several thousand fish in a small area. Zebrafish have been utilized as a laboratory species for quite some time so the optimum breeding and maintenance conditions have been well established (Westerfield 2000). The small size of zebrafish minimizes quantities of labware and chemicals, both for treating and maintaining live fish and for performing various assays (low quantities of reagents) and histological assessments (small amount of embedding materials and microscope slides) (Hill *et al.* 2002).

Big family

In contrast to mammals, zebrafish can generate large numbers of offspring. One pair of adult fish is capable of laying 200–300 eggs in one morning, and if kept under optimal conditions, they can provide this yield every 5–7 days. Additionally, as numerous fish are generally established for each genetic line, several pairs can be rotated to provide thousands of eggs daily and all year round. This can be improved by using newly matured fish that are between 3 and 6 months old (sexual maturation occurs around 100 days (Skidmore 1965)). In laboratory, it was reported a mean life span of zebrafish of 42 months, with the oldest individual surviving for 66 months (Gerhard et al. 2002). Despite this, the laboratory zebrafish are routinely kept for only 18 months to 24 months, after which they are considered to be of lower reproductive value.

Transparent and short childhood

Zebrafish embryos develop outside of the mother, allowing access to the embryos without the need to sacrifice the female. The eggs are big (diameter of about 1.0 - 1.5 mm), transparent and sturdy, allowing easy visualization of tissues or organs and allowing experimental manipulations, such as microinjections or transplantation of cells (Matthews et al. 2002; Dahm et al. 2006). As a vertebrate organism, the zebrafish presents many organs and cell types similar to that of mammals. Organogenesis occurs rapidly, and major organs (like brain, heart, liver, intestine and spinal cord) are present in larvae by 5 to 6 days post-fertilization (dpf) (Rubinstein 2003). Zebrafish development has been well characterized (Kimmel 1989; Kimmel et al. 1995).

Nobody is perfect

The differentiation between males and females is not always an easy task. As adults, males and females are of similar colouration, although males tend to have larger anal fins with more yellow colouration (Laale 1977) and females can be recognized by their larger belly accommodating the eggs (Dahm *et al.* 2006). As juveniles, the gender cannot be reliably distinguished without dissection and while gravid females have a more rounded body shape, the most reliable diagnostic feature is the presence of a small genital papilla in front of the anal fin origin (Laale 1977; Yossa *et al.* 2013).

When compared to other model organisms, such as the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, or the mouse *Mus musculus*, the number of tools and methods that are available when working with the zebrafish is still relatively limited (Dahm *et al.* 2006).

Studies with the zebrafish and radiation

With a better understanding of the biology of this animal, an increasing number of advantages and applications are claimed for its use in scientific research.

The impressive increase in the use of this animal model in scientific research can be understood by the analysis of the number of articles published in the last twenty two years. In order to facilitate this analysis, a graph based on scientific articles published on the PubMed database was created. Data used in the graph refers to works found between 1995 and 2016 in searches with the keyword "zebrafish" (Figure 7).

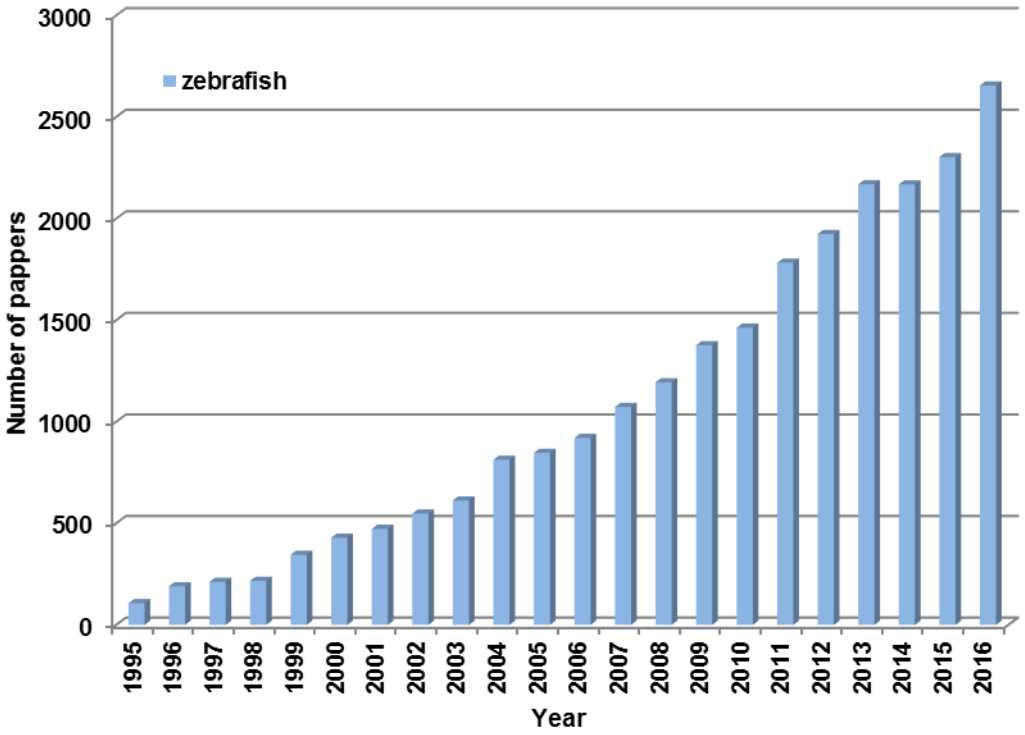

Figure 7 - Used Pubmed Query: Zebrafish [Title/Abstract] AND ("1995"[PDAT]: "3000"[PDAT]).

When we attune our search to articles that relate the use of this animal model and the study of the radiation everything changes. In fact, when we realize all the advantages listed about this animal, we began to recognize that their potential has not been really explored by radiobiology.

To prove that, we built a graph based on scientific articles published on the PubMed database which have the keyword "radiation, ionizing"[MeSH Terms] AND "zebrafish"[MeSH Terms] (Figure 8).

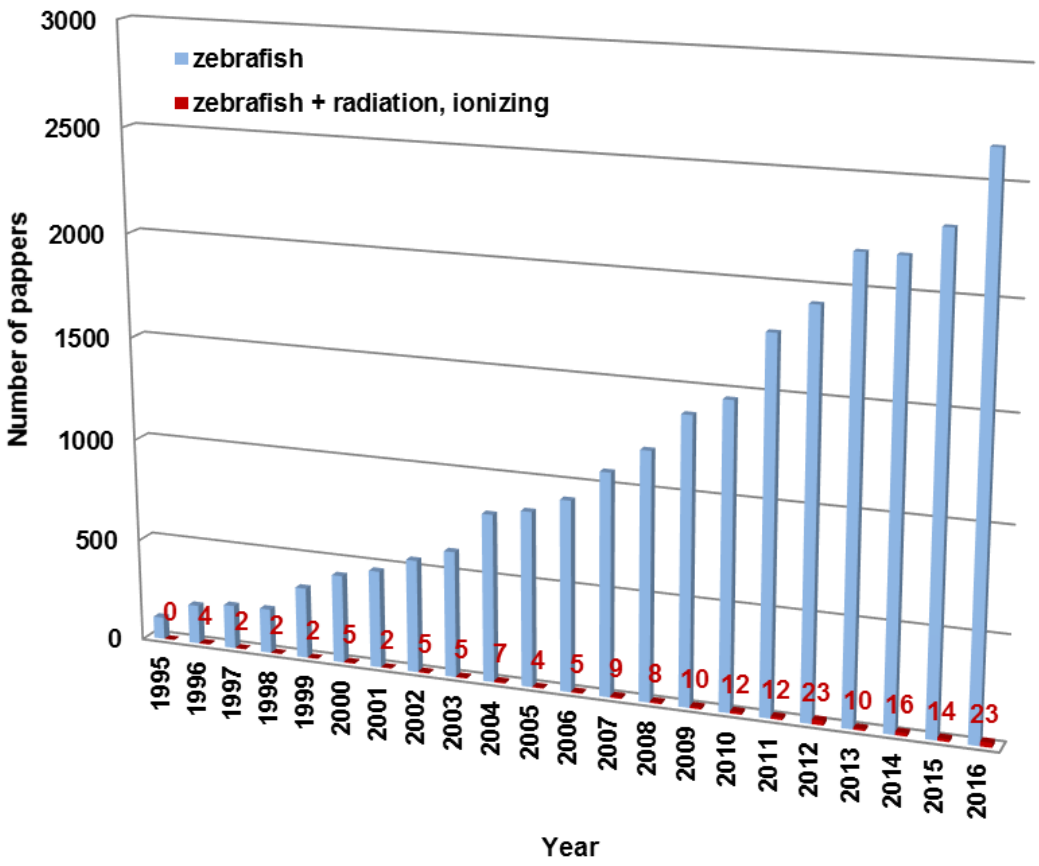

Figure 8 – Used Pubmed Query: "radiation, ionizing"[MeSH Terms] AND "zebrafish"[MeSH Terms] and “Zebrafish” [Title/Abstract] AND ("1995"[PDAT] : "3000"[PDAT]).

In general, of the articles published between 1995 and 2016 related to the zebrafish, only about 0.76% was somehow related to the use of ionizing radiation.

Despite its limited use, the zebrafish application as an animal model in radiobiology studies has given rise to very interesting and varied papers. In these studies, the zebrafish usually appeared as a model to:

- screen radiation modifiers;
- study radiation response *in vivo;*
- study bystander effects.

As a model to screen radiation modifiers

(McAleer *et al.* 2005; Daroczi *et al.* 2006; Geiger *et al.* 2006; Hwang *et al.* 2007)

The response of a cell to radiation can be changed by the action of certain chemical agents. These agents can reduce or improve the cell response. If the agents reduce the cell response to radiation, they are called radioprotectors and they generally influence the indirect effects of radiation by scavenging the production of free radicals. If the agents intensify the cell response to radiation, they are called radiosensitizer and they generally promote both the direct and indirect effects of radiation (Podgorsak 2005).

There are some works of researchers who have used the zebrafish to study new radioprotectors or radiosensitizers and there are also other studies that used well-known agents but that would never have been used in this animal model.

McAleer *et al.* (2005) evaluated the effects of ionizing radiation on morphologic development and survival of zebrafish in the absence and presence of a known radioprotector (free radical scavenger Amifostine) or a radiosensitizer (tyrosine kinase inhibitor AG1478). The aim of these experiments was to evaluate the zebrafish as a model of radiation response that may be used for easy and rapid testing of novel radiation protectors and sensitizers eventually intended for human therapeutic use. For this purpose, they exposed viable zebrafish embryos to X-rays (0, 2, 4, 6, 8, 10, 15 or 20 Gy) with or without either Amifostine or AG1478 at distinct developmental stages (1–24hpf). With regard to chemical agents used, amifostine markedly attenuated the radiation effect, while AG1478 enhanced teratogenicity and lethality, particularly at therapeutically relevant (2–6 Gy) radiation doses.

There are a number of other studies exploring the zebrafish as a model for the study of the action of radiation modifiers. Many of these studies are discussed in the review article published in 2007 by Hwang *et al*.

As a model to study radiation response *in vivo*

(Miyachi *et al.* 2003; Pereira *et al.* 2011; EPPERLY *et al.* 2012; Glass *et al.* 2013; Guo *et al.* 2013; Jaafar *et al.* 2013)

The zebrafish appeared as an alternative or a complement to *in vitro* techniques for the studying of the radiation response. One of the main limitations of the *in vitro* studies is related to the inability to reproduce (integrally) the complexity of cell microenvironment making it difficult to extrapolate the results to the *in vivo* processes.

Despite considering the zebrafish as a complete model, we cannot make a direct extrapolation to humans because, like in other animal models, its radiosensitivity is different from man. Many experimental irradiations show that mammals are the most radiosensitive, followed by birds, fish, reptiles, and insects (Linsley 1997). Considering LD_{50}, that is the dose that is lethal for half of the exposed individuals, the $LD_{50/60}$ (i.e. within 60 days) for human beings after whole-body radiation is around 4 Gy for persons managed without supportive care and 6 to 7 Gy when medical support are provided (Anno *et al.* 2003). On the other hand, the $LD_{50/60}$ for acute irradiation of marine fish is the range of 10-25 Gy (UNSCEAR 2008).

Aware of this limitation, there are many research groups who used the animal at its adult stage (EPPERLY *et al.* 2012; Glass *et al.* 2013) but there are also many others who took advantage of specific qualities of embryos (Miyachi *et al.* 2003; Pereira *et al.* 2011; Guo *et al.* 2013) , namely: (a) zebrafish embryos are optically transparent, which enables the microscopic assessment of embryogenesis development; (b) zebrafish embryos develop rapidly, reducing the duration of the experiments; (c) embryos can be produced in abundant number daily due to the high fecundity of zebrafish, which facilitates a high throughput of experiments; (d) zebrafish embryos can take up drugs (radioprotectors or radiosensitizers for example) directly from the medium, avoiding drug injections (Choi and Yu 2015).

Glass *et al.* (2013) studied the mechanisms of hematopoietic cell homing and engraftment in adult zebrafish since the radiation preconditioning is often used to prepare patients for hematopoietic cell transplant. They examined the zebrafish kidney that is the primary hematopoietic organ in zebrafish, and the use of radiation to precondition zebrafish for hematopoietic cell transplant of donor hematopoietic

cells obtained from this organ is well established. Knowing that the major complication of ionizing irradiation exposure is fibrosis, Epperly *et al.* (2012) demonstrated evidence for the efficacy of the zebrafish model in measuring radiation-induced long term effects and for screening small-molecule irradiation mitigators of late tissue fibrosis.

Miyachi *et al.* (2003) detected that zebrafish irradiated with low-dose X-rays tended to emerge earlier than controls, wherein the radiation appears to work as a stimulant. This observation led them to quantitatively examine the effects of low-dose X irradiation on a series of stages of development in the zebrafish. Still at embryonic level, Pereira *et al.* (2011) compared DNA damage induced by acute or chronic irradiation at cellular (zebrafish cell line ZF4) and developmental (embryo) levels. Both models, zebrafish ZF4 cells and embryos (at 3 h post-fertilization), were exposed to acute doses (0.3-2 Gy/d) or chronic dose rates (0.1-0.75 Gy/d). DNA damage was evaluated by immunodetection of γ-H2AX, DNA-PK and alkaline comet assay. Their results suggest a dose-dependent correlation between unrepaired DNA damage and abnormalities in embryo development, supporting the use of DNA repair proteins as predictive biomarkers of ionizing radiation exposure.

As a model to study bystander effects

(Mothersill *et al.* 2007; Smith *et al.* 2011; Choi *et al.* 2013; Kong *et al.* 2014; Choi and Yu 2015)

Bystander effect is ‘‘any effect induced in a cell as a result of another cell(s) being exposed to radiation’’ (Blyth and Sykes 2011). This phenomenon, which was demonstrated *in vitro* and *in vivo*, is categorized as a non-targeted effect of ionizing radiation. *In vitro* mechanistic studies of non-targeted effects of ionizing radiation revealed the important role played by the DNA repair pathways (Kadhim *et al.* 2013) and consequently the DNA repair mechanisms should also play an important role in *in vivo* situations too. Therefore, there is an increasing request for choosing an ideal animal model for effective studies of DNA repair mechanisms (Choi and Yu 2015). Pei *et al.* (2013) concluded that zebrafish represent an ideal model for studies on DNA damage and repair pathways since its genomic DNA contained orthologues of genes that are involved in all DNA repair pathways in higher eukaryotes and the role of specific DNA damage response genes in each repair pathway could be studied by simple morpholino-based or short hairpin RNA (shRNA) knockdown experiments. An additional advantage of zebrafish for these studies is that *in vivo* irradiation of fish should permit the presumed bystander factors to be secreted into the water making the chemistry involved in identifying the signal molecules easier (Mothersill *et al.* 2006).

Once again, this aspect can be studied either in adults or in embryos. Smith *et al.* (2013) investigated the bystander effect induction in adult fish of different species, namely zebrafish and medaka, irradiated with 0.5 Gy X-rays. Their results point to radiation induced bystander effect can transcend taxonomic group. In turn, Kong *et al.* (2014) studied *in vivo* radiation induced bystander effect between embryos of the zebrafish by alpha-particle irradiation, studying the number of apoptotic signals revealed at 24hpf.

Aims

The long term aim of this thesis was to explore the zebrafish as an animal model for radiobiology by assessing biomarkers at the cellular, biochemical and molecular levels and to gain knowledge about the mechanisms related to the *in vivo* responses to low doses of ionizing radiation. For these purposes, several animals were exposed to different doses (between 100 mGy to 1000 mGy) of X-rays, were sacrificed in different moments and samples of different organs were collected. An experiment with a comet assay protocol was applied to the blood and to the offspring (larvae) of the irradiated fish (Chaper 3). The antioxidant defenses and the oxidative damage were investigated in brain and liver (Chapter 4) and a study of the proteomic profiles was done in muscle tissue (Chapter 5).

As referred, the core of this thesis is organized in three main chapters where each of them is presented as an article after a General Material and Methods (Chapter 2):

Chapter 3. Single Low-Dose Ionizing Radiation Induces Genotoxicity in Adult Zebrafish and its Non-Irradiated Progeny

Chapter 4. Oxidative damage and antioxidant response in zebrafish liver and brain after exposure to low doses of ionizing radiation

Chapter 5. Assessing radiobiological effects of low doses of ionizing radiation on zebrafish muscle proteome

Chapter 2

General Material and Methods

Although in each chapter there is available the “Material and Methods” section for each experiment, it is pertinent to utter a few comments about some general points.

2.1. Animal maintenance

We used zebrafish as our animal model. Sexually mature one-year old zebrafish were retrieved from the *BOGA-CIIMAR (Biotério de OrGanismos Aquáticos - Centro Interdisciplinar de Investigação Marinha e Ambiental)*, an animal facility.

Fish were distributed by eight 28L-tanks (two for each group) within a recirculation water system (with dechlorinated tap water), equipped with a biofilter, aeration and heating, as presented in Figure 9.

In order to be sure that the number of males and females was correct, the identification of the gender of each animal was made using the method recommended by Yossa *et al.* (2013).

Water temperature was kept at 25±2°C and the photoperiod at 14h light/10h dark; fish density was inferior to 10 fish/L, as suggest by Reed *et al.* (2010). Fish were fed ad libitum twice a day with commercial flakes, but were fasted 24h prior to irradiation and euthanasia. Tanks were manually siphoned for cleaning whenever necessary. Animals were kept under these conditions for two months before beginning the experiment.

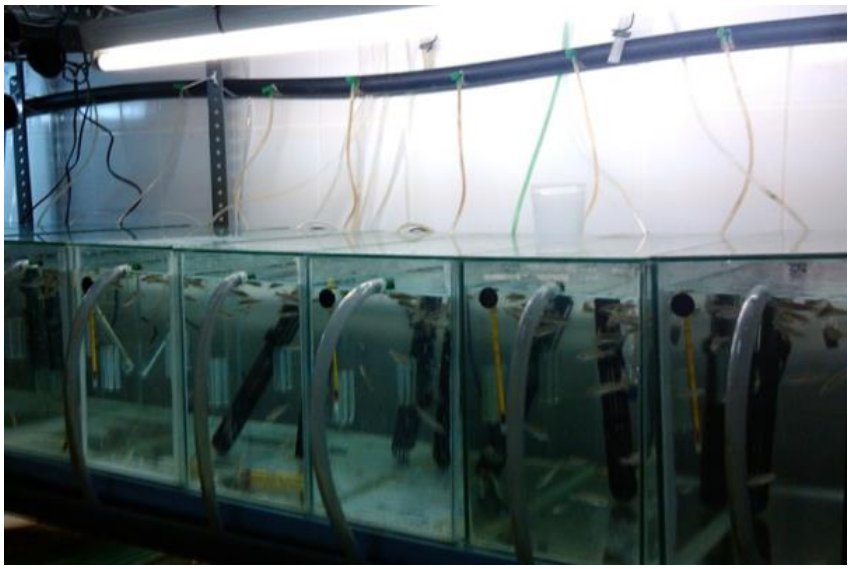

Figure 9 - Fish tanks.

2.2. Irradiation Procedure

Irradiations were performed in a 6 MV linear accelerator (Trilogy® System from Varian® Medical Systems). The animals were irradiated with 100 mGy (using 8 MU during 0.02 minutes), 500 mGy (42 MU, 0.1 minutes) and 1000 mGy (using 83 MU, 0.2 minutes). In order to confirm the desired dose an ionization chamber (PTW ®, TM30013, 0.6cc) was used. Animals were placed in a homogeneous field of 200 mL of water (1.5cm depth) and the irradiation took place at 1 meter away from the source, as shown in Figure 10.

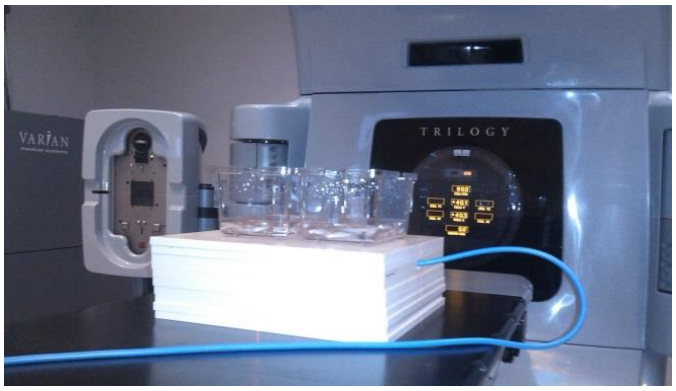

Figure 10 - Fish irradiation.

2.3. Animal Dissection

At day one, day four and day seven after irradiation, five animals of each group were rapidly euthanized by decapitation (making a total of 120 subjects – 60 females and 60 males).

It was collected peripheral blood sample for comet assay analysis (see Chapter 3). Livers, brains and muscles were excised, weighted, immediately frozen in liquid nitrogen and then stored at -80ºC for later biochemical analyses (see Chapter 4) and proteomics (see Chapter 5).

2.4. Animal care (3Rs)

The success of the zebrafish in biomedical research surely stems from following the '3Rs' principle: **Replacement**, **Reduction** and **Refinement**. This study complies with the principle of the 3Rs in the use of zebrafish for radiobiology through:

- **Replacement**: Animals (Scientific Procedures) Act 1986 (ASPA) refers, whenever possible, animals should be replaced by non-animal alternatives,

such as *in vitro* tests, to achieve the same scientific aim. It also includes the possibility to choose an animal with a “less complex” nervous system.

As a “lower vertebrate”, zebrafish is a good choice to replace mammalian models since there is less ethical concern for care fish in laboratories and it has less mental and physical concerns than rodents, for example.

In the specific case of radiobiology, it can be very difficult extrapolate from the results of *in vitro* work to the biology of the intact organism. Zebrafish can offer a cost-effective model to help bridge the “gap” between *in vitro* and *in vivo* work.

- **Refinement**: This experience was design to reduce stress, suffering, minimize potential pain, to improve welfare, husbandry and handling of animals since there is no alternative to the use of live animals in this research.

  Water quality is the central factor in maintaining the well-being of fish and in reducing stress and the risk of disease. Water-quality parameters were within the acceptable range that sustained normal activity and physiology for the specie. The groups consisted of fish of the same age and size to facilitate comparisons and to minimize the risk of injuries or cannibalism.

  Humane endpoints were determined for the experiments. A humane endpoint is defined by Organisation for Economic Co-operation and Development (OECD) as “the earliest indicator in an animal experiment of severe pain, severe distress, suffering, or impending death”. Once a humane endpoint is reached, the animal would be immediately euthanized. There was no endpoint achieved so all animals were killed in the originally predicted time (1d, 4d or 7d after irradiation).

  It was not administered any anaesthetic and analgesic because it could interfere with the study results.

  The housing animals and all tests were performed in CIIMAR facilities that are legally authorized to work with animal experiments. Everyone involved in this work are properly certified to handle animals.
- **Reduction:** The number of animals used was reduced through careful planning and proper experimental design, which allows used the smallest number of animals possible to obtain statistical significance.

Although the animals' eyes and gonads have not been analysed, these organs were dissected and stored at -80º for use in future experiments, so avoiding the use and sacrifice of more animals.

Chapter 3

Single Low-Dose Ionizing Radiation Induces Genotoxicity in Adult Zebrafish and its Non-Irradiated Progeny

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Single Low-Dose Ionizing Radiation Induces Genotoxicity in Adult Zebrafish and its Non-Irradiated Progeny

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Abstract This study investigated to what extent a single exposure to low doses of ionizing radiation can induce genotoxic damage in irradiated adult zebrafish (*Danio rerio*) and its non-irradiated F1 progeny. Four groups of adult zebrafish were irradiated with a single dose of X-rays at 0 (control), 100, 500 and 1000 mGy, respectively, and couples of each group were allowed to reproduce following irradiation. Blood of parental fish and whole-body offspring were analysed by the comet assay for detection of DNA damage. The level of DNA damage in irradiated parental fish increased in a radiation dose-dependent manner at day 1 post-irradiation, but returned to the control level thereafter. The level of DNA damage in the progeny was directly correlated with the parental irradiation dose. Results highlight the genotoxic risk of a single exposure to low-dose ionizing radiation in irradiated individuals and also in its non-irradiated progeny.

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Keywords Zebrafish · Ionizing radiation · Genotoxicity · Comet assay

Living organisms are chronically exposed to low doses of ionizing radiation in their environment. Natural sources (e.g. cosmic rays and radioactive substances in the earth's crust) are by far the major cause of this background radiation, but increasingly additional contributions have been given by anthropogenic sources over the past century (UNSCEAR 2010). Anthropogenic sources of radiation mainly include nuclear weapons use and testing, nuclear power production and accidents in nuclear power plants, as well as the use of radiation in medical procedures. The medical use of radiation has become the major man-made source of ionizing radiation exposure to humans, and is a growing concern for professionals of medical radiology due to occupational exposure (UNSCEAR 2010).

The DNA molecule is the primary target of ionizing radiation within the cell, and biological effects of radiation originate mostly from DNA damage. Ionizing radiation can induce DNA damage by changing the molecule chemical structure either directly or indirectly via radiation-generated reactive radicals (Harrison 2013). A multiplicity of radiation-induced DNA damages has been identified, including single-strand and double-strand breaks (Harrison 2013). If these damages are not efficiently repaired by naturally occurring DNA repair mechanisms, un- or mis-repaired DNA can lead to chromosomal abnormalities, gene mutations, cancer, and cell death. As a precautionary rule, it has been generally accepted that there is no safe dose of radiation—any amount increases the risk of damage (Mothersill and Seymour 2011; Duport et al. 2012). A linear no-threshold model has therefore been assumed for low-dose

radiation, stating that the risk of damage is directly proportional to exposure dose.

Considering the carcinogenic potential of low-dose ionizing radiation, several studies have focused on the effects of radiation at low doses in humans (Sari-Minodier et al. 2007; Ropolo et al. 2012; Saberi et al. 2013; Tug et al. 2013; Han et al. 2014) or have estimated the increased risk of cancer associated to such radiations (review in Prasad 2012). In general, these are epidemiological studies that compare retrospectively the incidence of a given effect in a selected group of previously exposed individuals (e.g. hospital radiology workers, patients irradiated for medical purposes, survivors from nuclear accidents) and a similar group of unexposed individuals. Although epidemiological studies in humans provide relevant information about health risks associated to low-dose ionizing radiation, they are subject to important constraints in terms of statistical power, uncontrolled variables, exposure misclassification, and selection bias (Duport et al. 2012). Experimental studies in cell cultures or laboratory animals, allowing working with populations with low individual variability and testing a wide range of accurate doses of radiation under strict control of all covariates, are a valuable alternative to assess the biological effects of radiation. On the other hand, since in vitro systems can respond differently to radiation comparing to in vivo systems (Jarvis and Knowles 2003; Bladen et al. 2007; Duport et al. 2012), experiments with laboratory animals can give us more precise insight into the effects of radiation and their underlying mechanisms.

Mammals (small rodents and dogs) are the most frequently used animals in experimental radiobiology (Duport et al. 2012). The zebrafish (*Danio rerio*) has become widely used as an in vivo model in many areas of biomedical research, but its utilization in radiobiology is still scarce and almost restricted to the embryonic stage. However, a number of favourable features, such as short generation time, easy reproduction and high fecundity, make this small teleost fish particularly suitable for studies on long-term and transgenerational effects of radiation. Moreover, since zebrafish and human genomes share a substantial degree of homology, including with regard to most DNA repair-related genes (Geiger et al. 2006), radiation studies in zebrafish can provide valuable information on radiation-induced human cancers. Finally, radiation studies in zebrafish, used as a model, can also be useful from an ecotoxicological point of view. In fact, aquatic ecosystems are prone to accidental or intentional contamination by radionuclides that undergo radioactive decay, resulting in the emission of ionizing radiation, whose impact on aquatic organisms must be evaluated (Matranga et al. 2010; Reinardy et al. 2011; Simon et al. 2011; Anbumani and Mohankumar 2012; Praveen Kumar et al. 2014; Saiyad Musthafa et al. 2014).

Experimental studies on the effects of low-dose ionizing radiation (up to 1 Gy) in living organisms have focused mostly on chronic exposure (from few hours to several months) and little attention has been paid to effects of single irradiation. In the present study we used the zebrafish as a biological model aiming at (1) investigating to what extent a single exposure to low doses of ionizing radiation, within the low-dose range for medical practice (≤1 Gy), can induce DNA damage in sexually mature individuals, and (2) evaluating the possibility of transmission of damage to the non-irradiated F1 progeny.

Materials and Methods

A group of sexually mature 1 year-old zebrafish purchased from a commercial supplier was kept in aquaria at water temperature of 25 ± 2°C and photoperiod of 14 h light/10 h dark, fed ad libitum twice a day with commercial flaked food, for about 2 months prior to irradiation. After that time, fish were sexed and distributed by four groups of 36 individuals, 18 of each sex. Three of these groups were externally irradiated with a single emission of X-ray at distinct doses: respectively 100 mGy (8 MU for 0.02 min), 500 mGy (42 MU for 0.1 min) and 1000 mGy (using 83 MU for 0.2 min); the remaining group was subject to the same handling as the other groups excepting that was not irradiated, serving as a control. The irradiation was performed using a Varian 6 MV linear accelerator, with fish placed inside a container with a homogeneous field of 200 mL of water (1.5 cm depth), at 1 m away from the beam source. An ionization chamber (0.6 cc PTW TM 30013) was used to confirm the desired doses. After irradiation, from each group, two couples of fish were housed in a separate appropriate cage for reproduction and the remaining fish were placed in an independent aquarium. A sample of five males and five females was taken from each aquarium/group at days 1, 4 and 7 post-irradiation to evaluate genotoxicity induced by radiation. Fertilized eggs of the first spawning from breeding couples of each group were collected, incubated until hatching, and five pools of five newly-hatched larvae (3 days post-fertilization) per group were sampled to evaluate genotoxicity in the progeny of irradiated parents.

Genotoxicity was assessed by measuring the level of DNA damage (DNA strand breaks) through the alkaline comet (single-cell gel electrophoresis) assay, performed according to Singh et al. (1988) and as previously described by Neuparth et al. (2013). In adult fish the effects were assessed in peripheral blood of each individual (5 males + 5 females per group and per sampling time): blood was collected just above the lateral line system with a syringe previously washed with 0.1 M EDTA to prevent clotting, and

blood cell suspensions were obtained by diluting (1:100) blood aliquots in cold homogenizing buffer (75 mM NaCl, 24 mM Na2EDTA, pH 7.5). In larvae the effects were assessed in whole body homogenates of pooled individuals (five pools per group): pools of five larvae were macerated in cold homogenizing buffer (75 mM NaCl, 24 mM Na2EDTA, pH 7.5), and homogenates were obtained by filtering through a 60 µm filter. Analysis was run immediately after blood collection (for adults) or whole body homogenization (for larvae) to ensure maximum cell viability. For that, 10 µL of blood cell suspensions or larval homogenates were diluted in liquid (37°C) 1% (w/v) low-melting point agarose and placed (2×75 µL) on microscopy slides previously coated with high melting point agarose. After the gel had set, the slides were placed into a cold lysing solution for 1 h (2.5 M NaCl, 100 mM Na2-EDTA, 10 mM Tris, pH 10, 1% Triton X-100, 10% DMSO). Slides were then placed in cold alkaline electrophoresis solution (0.1 mM Na2-EDTA, 0.3 M NaOH, pH 13), for 40 min. Electrophoresis was run for 30 min at 25 V using a horizontal gel electrophoresis tank. Slides were afterwards neutralized in cold Tris–HCl buffer (pH 7.5), and then dehydrated with ice-cold absolute methanol to be preserved until analysis. Before the examination, the slides were rehydrated and then stained with 0.02 mg/L ethidium bromide. A total of 100 randomly chosen cells were scored per slide under a fluorescence microscope and the comets were analysed using the software Comet Score 1.5 (TriTek Corp., Summerduck, USA). The percentage DNA in the tail, one of the most consensual and reliable comet metrics, was employed as a direct measure of DNA-strand breakage (Lee and Steinert 2003; Kumaravel and Jha 2006).

Data obtained with adult fish were firstly analysed by factorial (three-way) ANOVA, at the significance level of 0.01, to find if there was an interaction effect of factors (radiation dose, day post-irradiation, sex) on the magnitude of DNA damage. When a significant interaction occurred, one-way ANOVA was performed to identify the effect of one factor for each level of the remaining factors. In the case of larvae, since only one factor was studied (the radiation dose of parental exposure), data were analysed by one-way ANOVA. In both cases, when significant differences were detected by one-way ANOVA at the significance level of 0.01, means were compared by the Tukey multiple-range test. Analyses were performed using the software IBM SPSS Statistics version 22.

Results and Discussion

The radiation dose and the day post-irradiation had a significant overall effect on the level of DNA damage detected in irradiated adult zebrafish, contrarily to the sex of fish (Table 1). Moreover, a significant interaction effect of radiation dose and day post-irradiation was also observed (Table 1), meaning that the dose–response relationship was not the same in all days post-irradiation. Therefore, independent one-way ANOVAs were performed to compare the effect of dose in each day post-irradiation. For this, since both sexes responded identically, data obtained for males and females exposed to the same radiation doses in the same days post-irradiation were analysed together (5 females + 5 males, n = 10).

The level of DNA damage induced by radiation in adult zebrafish was positively dose-dependent at day 1 post-irradiation, and significantly higher in fish exposed to the highest dose than in control fish (Fig. 1). Relatively to day 1, at days 4 and 7 post-irradiation the level of DNA damage decreased in all irradiated groups and returned to the control level (Fig. 1).

It is well established that DNA strand breakage is a major early biological effect of ionizing radiation. Exposure of diploid mammalian cells to 1 Gy of radiation can generate about 1000 single- and 30 double-strand breaks per cell (Olive 2009). The alkaline comet assay is a rapid and sensitive technique to detect both kinds of DNA strand breaks (Collins et al. 2008; Collins 2015), and its usefulness in assessing DNA damage resulting from exposure to ionizing radiation has been well recognized (Kumaravel and Jha 2006; Collins et al. 2008; Olive 2009). Since the maintenance of DNA integrity is of chief importance, all living organisms developed efficient mechanisms for repairing DNA damage induced by genotoxicants. However, some DNA strand breaks can persist, depending, among other factors, on the repairing ability of the organism, on the dose of genotoxicant, and on the extent of exposure (Everaarts 1995; Shugart 2000). The return of DNA damage to the control level that we observed at day 4 post-irradiation can thus suggest the repairing of DNA strand breaks induced by radiation. Similarly, studies in tilapia (*Oreochromis mossambicus*) and rohu (*Labeo rohita*) showed a time-dependent

Table 1 Results of the factorial ANOVA to assess the effect of radiation dose, time after irradiation and gender on DNA damage of irradiated adult zebrafish

Source of variation	*df*	*F*	Significance
Radiation dose	3, 96	4.56	<0.01
Day post-irradiation	2, 96	10.87	<0.01
Sex	1, 96	6.17	>0.01
Radiation dose × sex	3, 96	1.32	>0.01
Day post-irradiation × radiation dose	6, 96	4.12	<0.01
Sex × day post-irradiation	2, 96	3.28	>0.01
Radiation dose × day post-irradiation × sex	6, 96	1.85	>0.01

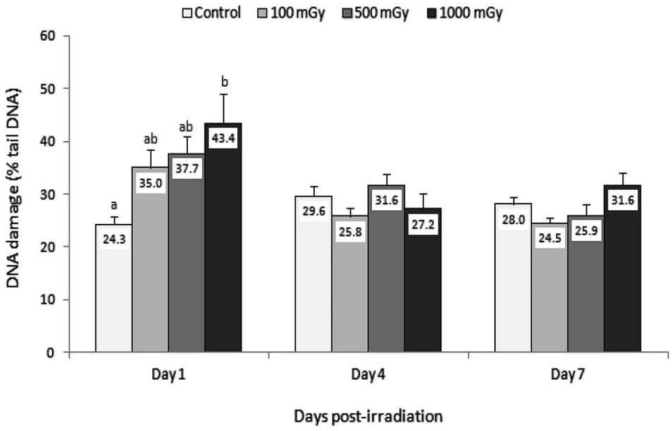

Fig. 1 Level of DNA damage in adult zebrafish at days 1, 4 and 7 post-exposure to different doses of ionizing radiation. Values are mean of n = 10; *error bars* indicate the standard error of mean. For each day, column values with *different letters* are significantly different ($p < 0.01$). Mean values of the control group at days 1, 4 and 7 are not significantly different

decrease of DNA damage detected by the comet assay in blood cells (and other tissues) after cessation of exposure to different toxicants, which was interpreted as the result of the DNA repairing activity carried out by fish (Banu et al. 2001; Ahmed et al. 2011; Mohanti et al. 2011). Previous findings, indicating high DNA repair capacity in zebrafish embryos (Sussman 2007) and rapid activation of genes associated with DNA repair, following induction of damage, in zebrafish larvae (Reinardy et al. 2013), seem to support our results.

The induction of genotoxic effects by ionizing radiation has been confirmed in different fish species, such as medaka (*Oryzias latipes*) (Kubota et al. 1995; Grygoryev et al. 2013) and Indian carp (*Catla catla*) (Anbumani and Mohankumar 2012, 2015). However, studies in these species have tested protracted or chronic exposure to radiation, in many cases at very high doses, instead of a single exposure to a low-dose radiation as in our study. In zebrafish, evaluation of genotoxic effects of ionizing radiation has been focused on early stages of development, i.e., embryos and larvae (Jarvis and Knowles 2003; Simon et al. 2011; Gagnaire et al. 2015), exposed to gamma-rays from very low to low doses (0.4–1000 mGy), for a variable period of time (1 h–20 days). DNA damage in early life stages of zebrafish were found at accumulated doses as low as 1.2 mGy (1.2 mGy/h for 1 h) (Jarvis and Knowles 2003), which represents an accumulated dose of about 100 times lower than the lowest single dose we tested in adult zebrafish. Although the exposure time may have influence when comparing these results, it is expectable that fish at early developmental stages are much more radiosensitive than adult fish. Actually, initial developmental stages are characterized by exponential growth and ongoing organ differentiation, with high rate of cell proliferation that renders DNA more vulnerable to radiation and mistakes of repair mechanisms.

In fish that were held to reproduce, spawning and subsequent fertilization occurred at the first day post-irradiation in the non-irradiated control group and at the third day post-irradiation in all irradiated groups. Since newly-hatched larvae were sampled three days after fertilization, this means that the level of DNA damage in larvae was assessed six days after parental irradiation. As spawning in zebrafish can be quite variable and no statistical analysis was performed due to lack of replicates, we cannot confirm if the spawning delay in irradiated groups was related to radiation exposure.

The level of DNA damage in the non-irradiated F1 progeny (newly-hatched larvae) was directly correlated with the radiation dose of parental exposure (Fig. 2), and reflected the initial level of DNA damage of the respective parents. Statistically, the level of DNA damage was significantly higher in offspring from parents exposed to the highest radiation dose than in the others (Fig. 2). Damages may have been transferred to the progeny through parental damaged germ cells, most likely through damaged sperm. Indeed, at least in mammals, there is evidence that post-meiotic male germ cells lose the ability to repair their DNA, contrarily to the oocyte (Harrouk et al. 2000). Thus, DNA lesions carried by the parental sperm may induce damage in the zygotes and in developing embryos, causing genomic instability that can persist through generations (Adiga et al. 2010). It has been suggested a delay in the activation of pathways inducing the genomic instability mediated by parental damages, retarding for a few days after fertilization the onset of the genotoxic response in the progeny (Adiga et al. 2010). Once this response is triggered, the intensive cell proliferation and differentiation that characterize developing embryos will lead to a high propagation of damaged

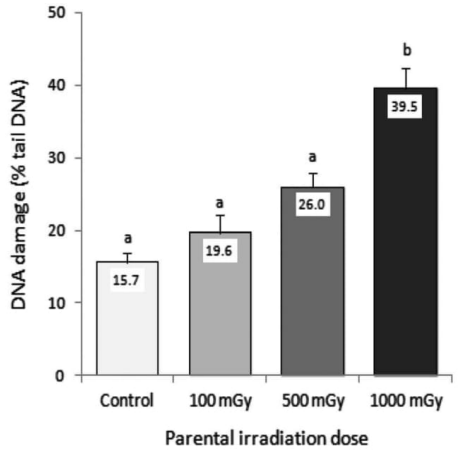

Fig. 2 Level of DNA damage in the unexposed progeny (newly-hatched larvae) of adult zebrafish exposed to different doses of ionizing radiation. Values are mean of n = 5; *error bars* indicate the standard error of mean. *Column* values with *different letters* are significantly different ($p < 0.01$)

cells. Considering that embryos also possess an efficient DNA repair capacity, this helps to explain the persistence of the relative high level of DNA damage in newly-hatched larvae, 6 days after parental irradiation, when damages in parents had already returned to the baseline. Comparable results were found in a study in mice, where comet assay was used to evaluate DNA damage in the sperm of irradiated males and its non-irradiated progeny (Adiga et al. 2010). In that study, the level of DNA damage in the sperm of the first-generation offspring also reflected the level of DNA damage in the parental sperm that, in turn, was radiation dose-dependent. Moreover, it was proved that the genetic damage was also transmitted to the somatic line of the progeny (assessed by increased percentage of micronuclei in fetal liver cells), following the same trend observed in the germ line. In our study, since homogenates of whole-larvae were used in the comet assay, damage found in offspring cannot be assigned to any particular cell type. Our results in zebrafish and those of Adiga et al. (2010) in mice support previous findings in medaka fish on the occurrence of mutations in the progeny of irradiated parents (Kubota et al. 1995; Shimada and Shima 2001, 2004). The transmission of genetic damages to future generations is responsible for transgenerational genomic instability, an important non-targeted, delayed effect of ionizing radiation (Barber and Dubrova 2006; Choi and Yu 2015).

Overall results highlight the genotoxic risk of a single exposure to low-dose ionizing radiation in irradiated zebrafish adults and also in its non-irradiated F1 progeny. Moreover, this work confirms the potential of zebrafish as an in vivo model in experimental radiobiology. Considering the present findings, further studies should be undertaken to provide insight into the transgenerational effects of ionizing radiation.

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Compliance with Ethical Standards

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Chapter 4

Oxidative damage and antioxidant response in zebrafish liver and brain after exposure to low doses of ionizing radiation

Abstract

Introduction: The use of zebrafish (*Danio rerio*) has grown considerably, demonstrating the increasing interest to use this model in biomedical research. This is essentially due to the level of homology shared with the human genome, complemented by an easy and reasonably affordable practical side. Hence, this work investigated radiobiological effects of exposure to low doses of ionizing radiation in zebrafish studying the activity of the antioxidant defence enzyme catalase (CAT) and the levels of lipid peroxidation (LPO) as an oxidative damage biomarker in liver and brain. Due to the increased usage of radiation in medicine, investigating potential effects of such exposure becomes a necessity inherent to public health, even when low doses are under consideration.

Material and methods: A population of 120 adult zebrafish has been used, with the animals divided in four groups of 30 each, with an equal number of males and females in each group. The control group suffered the same handling of irradiated fish but was not submitted to irradiation. The remaining three groups were externally irradiated with 100, 500 and 1000 mGy, respectively. Data was obtained from each group, with five males and five females sacrificed at one, four and seven days after irradiation, via brain and liver collection.

CAT is an antioxidant enzyme that catalyses decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. An increase in CAT activity reflects an increase in reactive oxygen species that may cause oxidative stress. Lipid peroxidation was assessed through the quantification of thiobarbituric acid reactive substances (TBARS). Both parameters were measured by spectrophotometric methods.

Results: In the brain, CAT activity varied between 1.62 to 5.55 μmol min^{-1} mg^{-1} protein and LPO level varied between 1.45 to 3.29 nmol TBARS mg^{-1} protein. In the liver, CAT activity varied between 43.02 to 80.37 μmol min^{-1} mg^{-1} protein and LPO level varied between 0.81 to 4.00 nmol TBARS mg^{-1} protein. Three-way ANOVA revealed significant effects of the interaction irradiation × gender × time for CAT activity and LPO level. Differential responses to dose of males and females were found for antioxidant and oxidative damage biomarkers, as indicated by significant dose × gender interactions. Significant dose × day post-irradiation

interactions were found for both biomarkers too, indicating that the genders showed different responses over time to the radiation dose.

Conclusion: Results obtained suggest that females and males present distinct responses over time to radiation. Our data point to a possible hyper-radiosensitivity in lower doses (≤500 mGy). Future studies should be targeted at clarifying factors responsible for the gender dimorphism observed and identifying other antioxidant and oxidative stress biomarkers involved in the response to low-dose IR exposure.

Keywords: zebrafish; X-rays; low doses; oxidative stress; catalase; lipid peroxidation.

4.1. Introduction

The biological effects of radiation in a living being result essentially from damage to DNA, proteins and lipids, which are critical targets within the cell (Charles 2001; Lombardi 2007; Pernot *et al.* 2012). After the irradiation of a cell, the damage can happen either directly or indirectly. By direct action, the radiation interacts directly with the critical target in the cell. The atoms of the target itself may be ionized or excited, leading to the chain of physical and chemical events that eventually produce the biological damage. On the other hand, by indirect action, the radiation interacts with other molecules and atoms (mainly water) within the cell to produce highly reactive molecules and free radicals derived from molecular oxygen that can, through diffusion in the cell, damage the critical targets within the cell (Evans and Halliwell 1999). After irradiation, these reactive oxygen species (ROS, e.g., oxygen ions, hydrogen peroxide, hydroxyl radical) are produced in cells over several minutes or hours (Riley 1994). Under such situations the cellular antioxidant system is stimulated so that the steady state level of ROS can be maintained. However, when ROS production surpasses the antioxidant capacity of the cell oxidative stress arises (Roots and Okada 1975). That is, an imbalance between the creation of free radicals and their removal by specific antioxidants (Riley 1994) will result in oxidative damage to cellular macromolecules, i.e., nucleic acids, proteins and lipids. Oxidative stress has been associated with many diseases (Thanan *et al.* 2014), like different cancer types (Wiseman and Halliwell 1996), Parkinson's disease (Tsang and Chung 2009), Alzheimer disease (Galasko and Montine 2010) and cardiovascular disorders (Suzuki *et al.* 2006). Therefore the organism must have protective mechanisms for the removal of excess free radicals (Noctor and Foyer 1998).

Investigation of oxidative responses in different *in vivo* models has found that cells, tissues and organs contain diverse antioxidant defence systems that form the basis for the differential susceptibility to oxidative environments (Si *et al.* 2013). Similar to what occurs in humans, aquatic organisms have developed a physiological antioxidant system reacting to ROS, which implicates antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR), and free-radical traps, such as glutathione (GSH) and vitamins E and C, among others. These components play an

important role in the protection against excess ROS-induced oxidative damage (Riley 1994; Si *et al.* 2013; Hou *et al.* 2015).

Antioxidant enzyme activities were frequently used to study reactions in fish to different aggression (Hou *et al.* 2015). Catalase, mostly located in peroxisomes (Evans and Halliwell 1999), is an antioxidant enzyme that catalyze decomposition of hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2) protecting the cell against oxidative stress induced by H_2O_2 or consequently formed hydroxyl (OH•) (Weydert and Cullen 2010; El-Bahr 2013). Lipid peroxidation (oxidation of polyunsaturated fatty acids) is a very important consequence of oxidative stress due to extensive production of ROS and/or reduced protective capacity of antioxidant system (e.g. catalase) (Boveris 2008). Malondialdehyde (MDA) is an indicator of oxidative damage that determines the level of lipid peroxidation (Benderitter *et al.* 2003).

Studies on biological effects of ionizing radiation using animal models are considered of extreme importance to overcome the limitations found when trying to extrapolate *in vitro* cell culture results to *in vivo* models. In recent years, the use of zebrafish (*Danio rerio*) has grown considerably, becoming ever more a very interesting model in biomedical research. This is essentially due to the availability of its complete genome sequencing, and the level of homology with the human genome, all this complemented by a quite easy and affordable practical side (Matthews *et al.* 2002; Spence *et al.* 2008). Based on these advantageous properties, its potential for radiobiological studies deserves each day more attention from researchers worldwide (Miyachi *et al.* 2003; McAleer *et al.* 2005; Geiger *et al.* 2006; Hwang *et al.* 2007).

The aim of the present study was to investigate the effect of ionizing radiation in adult zebrafish. Our main goal was to gain understanding of how low levels of ionizing radiation (100 mGy, 500 mGy and 1000 mGy) can induce antioxidant defences (namely CAT activity) and oxidative damage (by lipid peroxidation level evaluation) on brain and liver of adult zebrafish. The doses used in this study were chosen taking into account the application of ionizing radiation in medicine. According to a report from the National Council on Radiation Protection and Measurements (NCRP), people in USA were exposed to more than seven times as much ionizing radiation from diagnostic medical procedures in 2006 than they were in the early 1980s (Schauer and Linton 2009). Due to the increased usage of

radiation in medicine, investigating potential effects of such exposure becomes a necessity inherent to public health, even when low doses are under consideration.

4.2. Material and Methods

4.2.1. Animal maintenance and care

According to **Chapter 2.** General Material and Methods.

4.2.2. Irradiation Procedure

Three groups of fish were externally irradiated with a single emission of X-ray at distinct doses, respectively 100, 500 and 1000 mGy. A control group was subject to the same handling as the irradiated groups excepting that was not irradiated. Irradiation was performed according to **Chapter 2.** General Material and Methods.

4.2.3. Animal Dissection

At one day (1d), four days (4d) and seven days (7d) after the irradiation, ten animals of each group were euthanized by decapitation (making a total of 120 subjects – 60 females and 60 males). Livers and brains were excised and immediately frozen in liquid nitrogen and then stored at -80ºC for the analysis.

4.2.4. Biochemical analysis

CAT activity and LPO levels were determined in the brain and liver of zebrafish. The individual organs were homogenized in 100 mM phosphate buffer pH 7.4 (150 mM KCl, 1 mM Na_2EDTA and 1 mM DTT), and the suspension was then centrifuged at 12000 xg for 20 min at 4ºC.

The supernatant was used to determine LPO levels using the method described by Niki *et al.* (2000). Lipid peroxidation can be assessed through the quantification of thiobarbituric acid reactive substances (TBARS). This method is based on the reaction of compounds such as MDA, formed by degradation of initial products of free radical attack, with 2-thio-barbituric acid (TBA). The amount of TBARS was measured by reading absorbance at 532 nm ($\varepsilon = 1.4 \times 10^5\ M^{-1}cm^{-1}$) in a Bio Tek Power Wave 340 microplate reader. The results are expressed as nmol of TBARS per mg of protein.

CAT activity was determined by measuring the rate of disappearance of H_2O_2 according to Claiborne *et al.* (1985). Catalase acts on the conversion of hydrogen peroxide into water and oxygen. An increase in CAT activity reflects an

increase in oxidative stress imposed by the presence of H_2O_2. The decomposition of H_2O_2 was followed directly as the decrease in absorbance at 240 nm (ε = 40 M cm^{-1}); measurements were performed in a SHIMADZU 1603 UV/VIS spectrophotometer. The results are expressed as µmol min^{-1} mg^{-1} of protein.

Protein content in the samples was determined by the Coomassie blue method using bovine serum albumin (BSA) as a standard (Bradford 1976). All biochemical experiments were carried out in triplicate at 25 °C.

All chemicals and reagents used were of analytical grade and were purchased from Sigma–Aldrich Chemical (Steinheim, Germany).

4.2.5. Statistical analysis

All statistical analyses were performed using the software IBM SPSS Statistics 21 as described by Rodrigues *et al.* (2014). To investigate potential interactions between radiation dose, gender and day post-irradiation, data was analyzed by three-way Analysis of Variance (ANOVA) to assess significance of main effects and the four interaction terms for the two biomarkers (CAT and LPO). Significant dose x day post-irradiation x gender interactions could possibly indicate that dose x day post-irradiation was different in the two genders. Because interpretation of three-way interactions is complex, detailed analysis by two-way ANOVA was subsequently used to clarify the significance of the interaction term in each gender. A balanced design was always used, with the same number of replicates in each treatment and biomarker analyzed. The logarithmic transformation was applied to the data to fulfil ANOVA assumptions. Statistical significance was accepted for $P < 0.05$.

4.3. Results

The results of the biochemical determinations are presented below for brain and liver. Differences between genders were found in both tissues, as shown. Hence, data on temporal evolution of antioxidant responses and oxidative damage after irradiation is presented separately for males and females.

4.3.1. Effects of radiation on brain

CAT activity and LPO levels measured in the brain are presented in Table 3. For both parameters no significant differences were found among control groups at 1d, 4d and 7d, either in males or in females. In treated females CAT activity varied from 1.62 ± 0.37 (mean ± SD) to 5.47 ± 2.12 µmol min^{-1} mg^{-1} protein, found for the 100 mGy and 1000 mGy groups 4d after the irradiation, respectively. In treated males, activity levels ranged between 1.67 ± 0.37 and 5.55 ± 2.03 µmol min^{-1} mg^{-1} protein, found for the 100 mGy and 500 mGy groups 7d after irradiation, respectively.

Table 3 - CAT activity and LPO levels measured in the brain of zebrafish at 1d, 4d and 7d after exposure to low doses of ionizing radiation.

		CAT (µmol min^{-1} mg^{-1} protein)				**LPO** (nmol TBARS mg^{-1} protein)			
		Females		**Males**		**Females**		**Males**	
		Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
1d	Control	2.41	± 0.88	2.94	± 0.64	2.42	± 0.23	1.45	± 0.20
	100 mGy	1.70	± 0.40	2.68	± 1.55	1.85	± 0.12	1.97	± 0.54
	500 mGy	1.80	± 1.03	3.11	± 0.25	2.32	± 0.52	2.35	± 0.84
	1000 mGy	1.73	± 0.44	2.49	± 1.30	1.94	± 0.46	2.46	± 0.46
4d	Control	2.68	± 0.46	2.68	± 0.44	1.94	± 0.35	1.52	± 0.26
	100 mGy	1.62	± 0.37	1.87	± 0.72	1.92	± 0.20	2.13	± 0.49
	500 mGy	3.05	± 1.67	2.91	± 0.75	2.70	± 0.80	2.77	± 0.61
	1000 mGy	5.47	± 2.12	4.15	± 1.07	2.30	± 0.80	3.01	± 1.26
7d	Control	2.82	± 1.40	2.01	± 0.33	2.35	± 0.30	1.66	± 0.22
	100 mGy	3.52	± 1.11	1.67	± 0.37	3.10	± 0.41	2.65	± 0.45
	500 mGy	3.40	± 1.11	5.55	± 2.03	2.06	± 0.58	3.29	± 0.64
	1000 mGy	2.48	± 0.82	4.12	± 1.35	2.06	± 0.50	2.01	± 0.49

LPO levels in treated females varied on average between 1.85 ± 0.12 and 3.10 ± 0.41 nmol TBARS mg^{-1} protein, observed in 100 mGy group 1d and 7d after irradiation, respectively. In treated males, LPO levels ranged from 1.45 ± 0.20 to 3.29 ± 0.69 nmol TBARS mg^{-1} protein observed in the 1d control group and the 500 mGy group 7d after the exposure, respectively.

The three-way ANOVA revealed significant main effects of irradiation dose for CAT and LPO and time after irradiation for CAT activity (Table 4).

Table 4 - Results of the factorial ANOVA carried out to assess the effects of irradiation dose, time after exposure and gender on oxidative stress biomarkers determined in the brain of zebrafish.

Parameter		Source of variation	df	F	Sig.
CAT	Three-way ANOVA	Dose	3, 96	9.57	<0.001
		Gender	1, 96	3.29	0.073
		Day post-irradiation	2, 96	7.52	0.001
		Dose x Gender	3, 96	2.72	0.049
		Dose x Day post-irradiation	6, 96	6.72	<0.001
		Day post-irradiation x Gender	2, 96	4.04	0.021
		Dose x Day post-irradiation x Gender	6, 96	3.17	0.007
	Two-way ANOVA	Dose x Day post-irradiation (females)	6, 48	4.95	0.001
		Dose x Day post-irradiation (males)	6, 48	4.95	0.001
LPO	Three-way ANOVA	Dose	3, 96	7.74	<0.001
		Gender	1, 96	0.02	0.883
		Day post-irradiation	2, 96	2.49	0.088
		Dose x Gender	3, 96	6.66	<0.001
		Dose x Day post-irradiation	6, 96	2.82	0.014
		Day post-irradiation x Gender	2, 96	0.24	0.791
		Dose x Gender x Day post-irradiation	6, 96	1.57	0.164
	Two-way ANOVA	Dose x Day post-irradiation (females)	6, 48	2.97	0.015
		Dose x Day post-irradiation (males)	6, 48	1.58	0.175

Significant effects of the interaction dose × day post-irradiation × gender were observed for CAT activity, suggesting that the interaction between irradiation dose and time elicited different effects in males and females (Table 4). Detailed analysis by two-way ANOVA performed separately for each gender revealed statistical

significance of the interaction dose × day post-irradiation for both genders (Table 4). In females, differences over time were found for CAT activity in fish irradiated with 100 and 1000 mGy (Figure 11). In particular, opposite trends were observed; females exposed to 100 mGy showed lower activity, compared to controls, and then recovered to the control baseline. In females exposed to 1000 mGy CAT activity increased over time, then returning to control levels. In both cases, statistically significant differences were found after 4d of irradiation, two-folds higher than the control in the group treated with the highest dose (Figure 11). In males, significant two to three-fold inductions in CAT activity were found for fish irradiated with 500 and 1000 mGy, peaking at 7d after the exposure compared to controls (Figure 11).

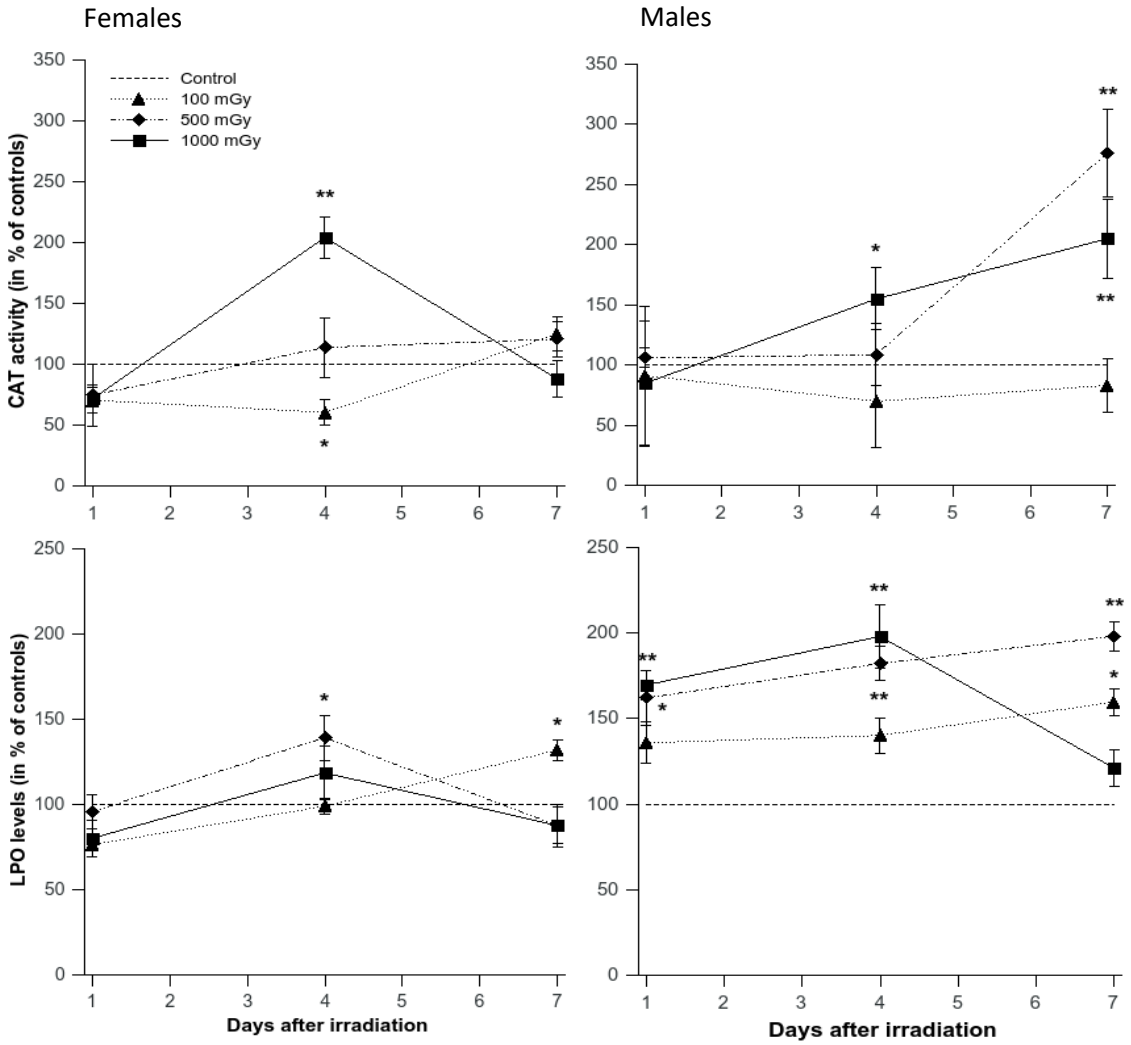

Figure 11 - Female and male variation in brain CAT activity and LPO levels (mean ± SE), relative to controls, at 1d, 4d and 7d after irradiation with low levels of ionizing radiation.
* Significant different from the control at $P < 0.05$ and ** significant different from the control at $P < 0.01$

Concerning the determination of LPO levels in the brain, the three-way ANOVA revealed significant interactive effects between dose and gender, and dose and day post-irradiation (Table 4). Upon detailed analysis by two-way ANOVA, it was found that the irradiation elicited different response profiles over time in the two genders (Table 4). The magnitude of changes in LPO levels, relative to controls, was clearly higher in males than in females. In the latter, alterations in LPO levels were only slightly above the control. In males, the strongest alterations were observed in fish irradiated with 500 and 1000 mGy. In the 500 mGy group LPO levels increased over time, peaking at 7d after the irradiation relative to controls (Figure 11). In the 1000 mGy group, compared to controls LPO levels increased until 4d after the irradiation and then decreased to baseline levels with differences no longer observed at 7d (Figure 11).

4.3.2. Effects of radiation in the liver

CAT activity and LPO levels measured in the liver are presented in Table 5. As expected, CAT activity was much higher in the liver than in the brain.

Table 5 - CAT activity and LPO levels measured in the liver of zebrafish at 1d, 4d and 7d after exposure to low doses of ionizing radiation.

		CAT (μmol min^{-1} mg^{-1} protein)		**LPO** (nmol TBARS mg^{-1} protein)	
		Females	**Males**	**Females**	**Males**
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
1d	Control	80.37 ±13.82	53.51 ±28.06	1.09 ± 0.39	1.51 ± 0.23
	100 mGy	70.13 ±19.75	53.29 ±15.84	0.99 ± 0.53	2.25 ± 0.74
	500 mGy	59.00 ±19.71	72.36 ±23.54	1.05 ± 0.31	1.93 ± 0.73
	1000 mGy	74.23 ±11.51	64.82 ±23.02	1.23 ± 0.79	3.69 ± 0.66
4d	Control	65.99 ±18.98	43.02 ±10.44	0.92 ± 0.62	0.89 ± 0.47
	100 mGy	51.40 ±22.53	58.68 ±23.38	2.23 ± 1.33	2.81 ± 1.36
	500 mGy	72.29 ±29.87	50.45 ±13.07	1.48 ± 0.73	4.05 ± 0.91
	1000 mGy	72.62 ±15.44	52.35 ±23.98	1.64 ± 1.12	2.49 ± 0.62
7d	Control	63.98 ±13.42	55.09 ±35.99	0.81 ± 0.48	1.15 ± 0.40
	100 mGy	71.23 ±7.60	58.77 ±22.55	1.34 ± 0.85	1.54 ± 0.87
	500 mGy	64.71 ±20.36	64.40 ±19.64	2.19 ± 1.27	2.73 ± 0.66
	1000 mGy	68.06 ±11.00	48.14 ±27.01	2.26 ± 1.69	4.00 ± 1.86

In females, CAT activity varied between 59.00 ± 19.71 and 80.37 ± 13.82 μmol min^{-1} mg^{-1} protein observed in the 500 mGy treatment and in controls one day

after irradiation, respectively (Table 5). In males it ranged from a minimum average of 43.02 ± 10.44 µmol min^{-1} mg^{-1} protein, found in controls four days after irradiation, to a maximum of 72.36 ± 23.54 µmol min^{-1} mg^{-1} protein, found in the 500 mGy treament in the day after irradiation. In the three-way ANOVA only a significant main effect of gender could be identified (Table 6). Detailed two-way ANOVA also showed no significant effects of irradiation dose or time after irradiation on CAT activity of either females or males (Table 6, Figure 12). Measured values of irradiated groups were generally near control values.

Table 6 - Results of the factorial ANOVA carried out to assess the effects of irradiation dose, time after exposure and gender on oxidative stress biomarkers determined in the liver of zebrafish.

Parameter		Source of variation	df	F	Sig.
CAT	Three-way ANOVA	Dose	3, 96	0.40	0.754
		Gender	1, 96	10.34	0.002
		Day post-irradiation	2, 96	1.00	0.372
		Dose x Gender	3, 96	1.59	0.198
		Dose x Day post-irradiation	6, 96	0.33	0.919
		Day post-irradiation x Gender	2, 96	0.08	0.922
		Dose x Gender x Day post-irradiation	6, 96	0.73	0.629
	Two-way ANOVA	Dose x Day post-irradiation (females)	6, 48	1.21	0.318
		Dose x Day post-irradiation (males)	6, 48	0.30	0.935
LPO	Three-way ANOVA	Dose	3, 96	14.22	<0.001
		Gender	1, 96	34.80	<0.001
		Day post-irradiation	2, 96	1.24	0.329
		Dose x Gender	3, 96	2.28	0.084
		Dose x Day post-irradiation	6, 96	3.28	0.006
		Day post-irradiation x Gender	2, 96	1.15	0.322
		Dose x Gender x Day post-irradiation	6, 96	1.20	0.315
	Two-way ANOVA	Dose x Day post-irradiation (females)	6, 48	1.26	0.294
		Dose x Day post-irradiation (males)	6, 48	3.71	0.004

LPO levels tended to be lower in the liver than in the brain. In females, LPO levels ranged between 0.81 ± 0.48 and 2.26 ± 1.69 nmol TBARS mg^{-1} protein, found respectively in the controls and the 1000 mGy group seven days after irradiation. In males the values varied between 0.89 ± 0.47 nmol TBARS mg^{-1} protein, found for controls four days after irradiation, and 4.00 ± 1.86 nmol TBARS mg^{-1} protein, found in the 1000 mGy group seven days after irradiation (Table 6). The three-way ANOVA indicated significant effects of irradiation dose and gender, as well as the interaction between dose and day post-irradiation after irradiation on LPO levels, suggesting that effects triggered by irradiation and their temporal evolution would be different between females and males. Detailed two-way ANOVA confirmed a significant interaction between dose and day post-irradiation for males but not for females (Table 6). In the latter, increasing trends over time could be observed in the 500 and 1000 mGy groups, but variability was high so that no differences compared to controls could be detected.

In males the two-way ANOVA showed that different irradiation doses elicited different response patterns over time, as indicated by the significance found for the interaction between dose and time (Table 6). No significant differences could be found among controls groups at 1, 4 and 7 days. Average values in irradiated groups were above control levels. Significant increases in LPO levels were detected for all irradiation doses, which were about 2.5 to 4.5 folds higher than controls (Figure 12).

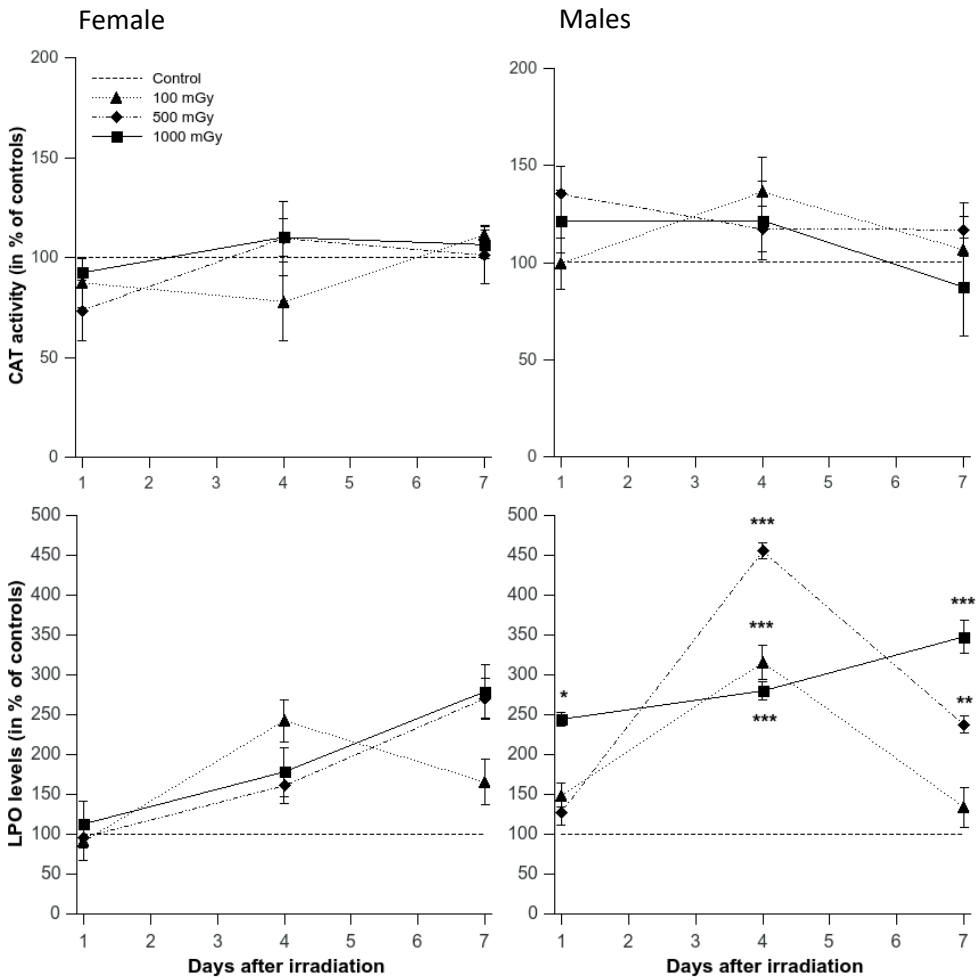

Figure 12 - Female and male variation in liver CAT activity and LPO levels (mean ± SE), relative to controls, at 1, 4 and 7 days after irradiation with low levels of ionizing radiation. * Significant different from the control at $P < 0.05$ and ** significant different from the control at $P < 0.01$

Fish exposed to 100 and 500 mGy showed very high increases in LPO from the first to the fourth day after irradiation, starting to decrease thereafter. Those irradiated with 100 mGy reached control levels at seven days (Figure 12). In those irradiated with 500 mGy LPO was so altered at the fourth day that, despite the strong decrease subsequently noted, LPO levels remained about 2.5 folds higher, relative to controls. Fish irradiated with 1000 mGy showed a completely different pattern. LPO levels were already 2.5 fold higher than controls one day after irradiation and continued to increase until seven days after irradiation.

4.4. Discussion

It is known that ionizing radiation is a potent inducer of oxidative stress (Spitz *et al.* 2004; Azzam *et al.* 2012). This type of radiation produces ROS as the result of primary interaction with tissue and as a secondary consequence of biological injury (Suzuki *et al.* 2002; Zhao and Robbins 2009). ROS can interact with critical biomolecules of the cell, induce oxidative stress (imbalance of pro-oxidants versus antioxidants) and cause damage to these macromolecules, compromising cell viability (Goldberg and Lehnert 2002; Marnett *et al.* 2003). This can contribute to the progression of multiple diseases (Karihtala and Soini 2007; Kregel and Zhang 2007; Thanan *et al.* 2014). Since the production of ROS is a normal aspect of cellular metabolism - even in the absence of radiation exposure - the interpretation of ROS role in radiation damage is a complex process (Kowaltowski *et al.* 2009).

In this study, catalase activity was much lower in the brain than in the liver, both in females and males. This is consistent with the high susceptibility of the brain to oxidative damage. This organ has relatively low antioxidant system, despite its high ROS production rate (Mates 2000; Verstraeten *et al.* 2008). Major factors explaining this imbalance are its high rate of oxygen consumption, the high rate of oxidative metabolism, the rich content in oxidizable polyunsaturated fatty acids of brain membranes and the lower activity levels of antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase, compared to other tissues (Verstraeten *et al.* 2008). All this contributes for the key role of ROS in damage originated in neurodegenerative processes, including cell-death, motor neuron diseases and axonal injury, among others. Together with the present results, such characteristics indicate that both the brain and liver should be considered in future biomarkers investigations concerning mechanisms of action and effects of ionizing radiation.

Different response patterns to whole body low-dose exposure were also observed for females and males. Concerning to females, the higher dose (1000 mGy) stimulates the antioxidant defence system in brain on the day fourth after irradiation. CAT activity dropped and metabolic activities gradually normalized within 7 days after exposure. In agreement with these alterations, no significant lipid peroxidation was found at this dose level, suggesting the antioxidant defence system was able to cope with the ROS increase triggered by the exposure. A

different activity profile was found for lower doses. CAT inhibition or no alteration relative to controls was found in the female brain after irradiation with lower doses (100 and 500 mGy). In contrast, increases in LPO were found in fish irradiated with 500 mGy and 100 mGy at 4 and 7 days, respectively. Decreases in CAT could result from the flux of superoxide radicals, which have been reported to inhibit the enzyme activity (Kono and Fridovich 1982). Moreover, LPO augmentation suggests that the low CAT levels were insufficient to deal with the exposure.

In the female liver, CAT activation was never observed independent of the radiation dose applied or the time after irradiation. However, the increased LPO variability, relative to controls, with a tendency for augmented damage, is a recognized effect of exposure to chemical stressors. Overall, the results obtained in the lower doses suggest that females may exhibit radio-hypersensitivity (Marples 2004; Marples and Collis 2008), which decreases and disappears as a result of the activation of repair systems for doses higher than 500 mGy (Tubiana *et al.* 2006). Over the last decade, a region of high sensitivity in the radiation survival response of mammalian cells was identified at doses below 500 mGy (Joiner *et al.* 2001). Hypersensitivity has been associated to bystander effect where damage occurs not only to the cell that was exposed to radiation but also to surrounding cells. This model gives reasons to state that the radiation is more damaging than previously thought. The mechanisms related with the radiation induced bystander effect are not fully understood but gap-junction intercellular communication (Mancuso *et al.* 2008), the secretion of soluble factors from irradiated cells to the bystander cells (Khan *et al.*) and the reactive oxygen species generated were found to play important roles. Since the bystander signal also induces an elevation in intracellular levels of ROS, including superoxide and hydrogen peroxide (Lorimore *et al.* 2003) it can be an explanation to CAT decrease.

Relating to males, different response patterns were also found among treatments, which point to an important role of CAT activity in protecting against oxidative damage to membrane lipids and radio-hypersensitivity. Irradiation with 1000 mGy led to sustained increase over time in CAT activity, able to prevent LPO at seven days. Low-dose irradiation (100 and 500 mGy) resulted in no activation or delayed activation of the enzyme, which was accompanied by higher LPO levels compared to controls. Likewise, in the male liver, activation of CAT was ever found and LPO showed increases of about 2.5 to 4.5 folds in all irradiation doses at most

of the time points. The threshold for CAT activation thus appears to be higher in the liver, compared to the brain. It is of note here that the liver is the organ with the highest content in antioxidant enzymes and molecules. However, relative to females, such high differences in LPO raise the hypothesis that males would show a wider range of hypersensitivity and could also be more susceptible to the bystander effect.

In this investigation, it would appear that the ROS generated from low-dose irradiated cells were at the level of reported bystander signals that could lead to oxidative stress, increasing damage. This subject is controversial, however, as other authors reported that the ROS production upon radiation exposure could also activate the synthesis of antioxidants (Kojima *et al.* 2011). This stimulation of defence mechanisms would in turn allow for a radioadaptive response (McDonald *et al.* 2010), protecting cells from subsequent radiation. However, *in vitro* studies suggested that radioadaptive response might not have the same mechanism than toxic bystander effect (Ryan *et al.* 2009). Differences in genetic and epigenetic factors could be at the origin of the dual character of the radiation-induced bystander effect (Mothersill 2012). Effects of low-dose irradiation on brain (Yamaoka *et al.* 1991; Kojima *et al.* 1998) and liver (Yamaoka *et al.* 1991; Kojima *et al.* 1997; Kojima *et al.* 1998; Avti *et al.* 2005) antioxidant defence systems were investigated previously in rodents. Though the studies employed different radiation doses, with different dose rates and time intervals in another animal model, they suggested that enhancement in the levels of GSH and antioxidant enzyme activities (e.g., SOD and CAT) in these organs would result in a hormetic/adaptive response of the cells. Another research group studied effects of occupational exposure to low doses of ionizing radiation in the antioxidant status of the radiology staff. The study population included 70 subjects and the doses of exposed staff ranged between 0.10 and 3.8 mGy per month. Their results showed that the activities of erythrocyte copper zinc-superoxide dismutase (CuZn-SOD) and selenium dependent glutathione peroxidase (Se-GPx) enzymes observed for the exposed group were significantly higher than in the controls. On the other hand, the activity of CAT enzyme and MDA levels were significantly lower in the exposed group than in the controls. The study thus suggested that a stimulant effect of chronic low-dose radiation would occur in exposed individuals potentially resulting in enhanced resistance to oxidative stress (Eken *et al.* 2012)**.**

The dimorphism observed may be due to differential hormonal regulation in males and females. Otherwise, it may be related to gender differences in susceptibility to oxidative stress or mitochondrial bioenergetics. Previous studies showed that Drosophila females, for example, tended to have higher levels of hydrogen peroxide production and significantly lower levels of catalase but not superoxide dismutase compared to males (Ballard *et al.* 2007). Yet, mammalian females tend to have lower rates of ROS production and higher antioxidant activity. In both type of organisms, female mitochondria consume higher quantity of oxygen when provided with adenosine diphosphate and have greater mtDNA copy number than males (Ballard *et al.* 2007). Considering that zebrafish is also a vertebrate and several mechanisms are highly conserved relative to mammals, this would additionally explain the higher radioresistance of females, compared to males, found in the present study.

4.5. Conclusions

The study results highlighted the need to include the brain and liver in future investigations of ionizing oxidative stress biomarkers and mechanisms. Distinct responses to radiation doses over time were identified for males and females. The data point to possible hyper-radiosensitivity to low-dose exposures (≤500 mGy) in males and females. However, male liver appears to show wider range of hypersensitivity as well as possible increased susceptibility to the bystander effect. Future studies should be targeted at clarifying factors responsible for the gender dimorphism observed and identifying other antioxidant and oxidative stress biomarkers involved in the response to low-dose ionizing radiation exposure.

Chapter 5

Assessing radiobiological effects of low doses of ionizing radiation on zebrafish muscle proteome

Abstract

Introduction: This paper relates with the application of zebrafish – *Danio rerio* – to the study of radiobiological effects of low doses of ionizing radiation. In recent years, the use of zebrafish has grown considerably, pointing more and more as a very interesting model in biomedical research, essentially because of the level of homology shared with the human genome, complemented by an easy and reasonably affordable practical side. The two-dimensional gel electrophoresis (2DE) followed by matrix-assisted laser desorption/ ionization - time of flight (MALDI-TOF) mass spectrometry is a method used for the analysis of complex protein mixtures from biological samples. This combined technique allows the detection of differences in protein expression and an overview of the proteome of zebrafish under different conditions, so allowing to infer the metabolic status and interpret the physiology of zebrafish. The present work aimed at evaluating proteome changes in the zebrafish muscle caused by low doses of X-rays exposure.

Material and Methods: The animals were externally irradiated with three distinct dose protocols (100 mGy, 500 mGy and 1000 mGy). At one day after the irradiation, the animals were euthanized and a sample of the muscle was collected. 2DE and MALDI-TOF mass spectrometry were used to characterize the muscle proteins that showed a differential expression after X-rays exposure.

Results and Discussion: An average of 85 protein spots from zebrafish muscle were separated and detected by 2DE. The statistical analysis retrieved differences in the expression of 32 proteins between the control and the irradiated groups. Most of these proteins were down-regulated in irradiated groups when compared to the control group. Among the 15 identified proteins that changed after irradiation are those involved in cytoskeleton structure, ubiquitin-dependent protein catabolism, kinase dependent regulation of signal transduction, lipid transport and a protein with a putative function in cell differentiation and regulation of apoptosis.

Conclusions: We confirm that the 2DE and MALDI-TOF method are adequate analytical tools for study radiobiological effects at the molecular level in zebrafish. Several proteins from the muscle of zebrafish were down-regulated suggesting that a remodelling of the muscle proteome occurs after the exposition to low doses of X-rays. These protein abundances alterations may prejudice muscle function that might result for example in abnormal swimming of zebrafish.

Keywords: zebrafish; ionizing radiation; low doses; radiobiology; proteomic.

5.1. Introduction

Nowadays the exposure to medical radiation is the greatest manmade source of radiation exposure to the general population (UNSCEAR 2008). According to a National Council on Radiation Protection and Measurements (NCRP) report, the Americans were exposed to more than seven times as much ionizing radiation from diagnostic medical procedures in 2006 than they were in the early 1980s (Schauer and Linton 2009).

Despite of the biological effects of radiation at high doses being well documented (Committee to Assess Health Risks from Exposure to Low Levels of Ionizing Radiation 1998; Hall 2001; Preston *et al.* 2007; Harley 2008), the risks and long-term impact of intermittent or continuous exposure to low-dose radiation on human health are still unclear (Pernot *et al.* 2012). Therefore, given the increasing number of medical procedures using radiation and the uncertainty of the real biological effects of low doses of radiation, more studies are imperative to shed more light on this problem.

Studies on biological effects of ionizing radiation using animal models are considered of extreme importance to overcome the limitations found when trying to extrapolate *in vitro* cell culture results to *in vivo* models. In recent years, the use of zebrafish (*Danio rerio*) as a model in biomedical research has grown considerably, essentially because of the full knowledge of its genome and the level of homology with the human genome, all this complemented by a quite easy and affordable practical side (Matthews *et al.* 2002; Spence *et al.* 2008). Based on these advantageous properties, its potential for radiobiological studies deserves each day more attention from researchers worldwide (Miyachi *et al.* 2003; McAleer *et al.* 2005; Geiger *et al.* 2006; Hwang *et al.* 2007). In these contexts, the response of zebrafish to X-rays may help illuminate the effects of this exposure on humans.

Proteins are the major catalytic and structural components in all living systems, and thus have a key role in all cellular and physiological processes, and in the organism response to systematic perturbations (Leszczynski 2013). Therefore, proteomic analysis is a powerful tool to investigate the cellular response to injuries, such as those caused by ionizing radiation. The two-dimensional gel electrophoresis (2DE) is the standard method used for the analysis of complex protein mixtures and proteomes. This method also allows the detection of quantitative protein differences

and comparative analysis being therefore appropriate to study the effects of irradiations at the proteome level (Azimzadeh *et al.* 2014; Leszczynski 2014). In this study we used 2DE method to characterize the differential expression zebrafish exposed to low doses of X-rays, followed by identification of altered proteins by MALDI-TOF mass spectrometry.

Our main goal in the current study was to characterize the differential protein expression in the muscle of male zebrafish exposed to medical low doses of X-rays.

5.2. Material and Methods

5.2.1. Animal maintenance and care

According to **Chapter 2.** General Material and Methods.

5.2.2. Animal exposure to radiation

Three groups of fish were externally irradiated with a single emission of X-ray at distinct doses, respectively 100, 500 and 1000 mGy. A control group was subject to the same handling as the irradiated groups excepting that was not irradiated. Irradiation was performed according to **Chapter 2.** General Material and Methods.

5.2.3. Animal Dissection

At one day after the irradiation, four males (n=4) of each group (control, 100 mGy, 500 mGy and 1000 mGy) were euthanized by decapitation (making a total of 16 subjects). A sample of the muscle was excised and immediately frozen in liquid nitrogen and then stored at -80ºC for the analysis. We used the muscle tissue since it is easy to sample (which reduces the experience errors), is the most abundant tissue in zebrafish and is relatively homogenous compared to other organs. Besides has a large protein fraction (which is very important considering the animal size) and there is a considerable literature on the skeletal muscle proteome (Bosworth *et al.* 2005).

5.2.4. Sample preparation

Every time that the frozen sample was handled, it was used a styrofoam box with ice to avoid protein degradation.

Muscle samples were homogenized in a buffer solution (7M Urea, 2M Thioureia, 4% CHAPS, 65mM DTT and 0,8% v/v ampholytes, in distilled water) with protease inhibitors (Halt™ Protease inhibitor cocktail, EDTA free, diluted 100x), in a ratio of 0.215 g fresh weight tissue per 1 mL of buffer according to the method described previously (Campos *et al.* 2013). The samples were sonicated three times during 5 seconds followed by 1 minute cooling on ice after each sonication and then centrifuged at 16 000 x g for 10 minutes at 4ºC. The supernatants were collected and stored at -80ºC until further analysis.

Protein was determined according to the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as a standard. Briefly, 5 µL of the diluted samples were mixed with 250 µL of a commercial Coomassie Brilliant Blue G 250 solution (B6916, Sigma) and after 30 minutes, the absorbance was read at 595 nm in the spectrophotometer. All experiments were carried out in duplicate.

5.2.5. Two-dimensional gel electrophoresis

Two-dimensional electrophoresis was performed based on a previous established protocol (Campos *et al.* 2013; Valerio *et al.* 2014).

5.2.5.1. First dimension – isoelectric focusing (IEF)

Each sample (500 µg protein) was diluted in rehydration buffer to a final volume of 300 µL. Samples were thereafter loaded in IEF gel strips with 17 cm length and linear pH gradient from 4 to 7, in a IEF electrophoresis tray.

Mineral oil (4 mL) was placed on top of each IEF gel strip just before the electrophoresis. The proteins were then separated in a Protean IEF Cell (Bio-Rad, EUA, California), at constant temperature (20ºC) employing the following program: gel strip rehydration - 12h, 50V; Step 1 – 15min at 250V; Step 2 - 3h voltage gradient to 10000 V (linear ramp); Step 3 – 10000 V/h until 60000 V; Step 4 – hold on 500 V.

Once the IEF was over, the strips were removed from the equipment, transferred to a strip holder tray and stored at -20 ºC until performing the second dimension electrophoresis.

After the IEF and before the second dimension it was necessary to equilibrate the IEF gel strips in two steps (Gorg *et al.* 2000): Step 1 – equilibration buffer with 10 mM DTT; Step 2 – equilibration buffer with 25 mM iodoacetamide (Biorad, USA, California). The equilibration buffer was prepared with 50 mM Tris-HCl, pH 8.8, 6M urea, 30% (v/v) glycerol, 2% (w/v) SDS and bromophenol blue. Each gel strip was incubated with 2.5 mL of equilibration buffer for a period of 15 minutes and shaking.

5.2.5.2. Second dimension- SDS-PAGE

The second dimension consisted of a vertical polyacrylamide (12,5%) gel electrophoresis under denaturing conditions (SDS-PAGE).

Electrophoresis was performed in the Hoefer SE900 system (Hoefer, USA, Massachusetts). This electrophoresis system allowed the simultaneous running of 6 polyacrylamide.

After the equilibration, the IEF gel strips were assembled in SDS-PAGE gels together with molecular weight markers (Bio-rad). Proteins were separated by SDS-PAGE at constant electric current (480 mA) for a period of about 6 hours (until the bromophenol dye front reached the end of the gel).

5.2.5.3. Gel staining

For visualization of proteins SDS-PAGE gels were stained with Colloidal Coomassie Blue according to Neuhoff *et al.* (1988) and Heinemeyer *et al.* (2007).

5.2.5.4. Gel image acquisition

The 2DE gel images were obtained using a calibrated scanner GS-800 (Bio-rad, USA, California) and the Quantity One software (Bio-rad, USA, California). The high spot resolution areas of the gels were cropped and background subtracted, before conducting the expression analysis.

5.2.5.5. Protein expression analyses

In total 16 2DE gels were performed corresponding to the biological samples (n=4) that were collected from each of the 4 experimental groups. Protein spot intensities were normalized to the total density in the gel image and a master gel (a synthetic image containing the spot data from all gels) was created in the PDquest software (Biorad). After optimizing the image display, the spot detection and matching were manually improved.

The Student's t-test ($P \leq 0.05$) was used to observe variations in the intensity (relative abundance) of each protein spot between the control and the exposed groups.

5.2.6. In-gel digestion of proteins and MALDI-TOF analysis

Protein spots with significant variations in expression were excised from gels, destained, washed, dried and digested using the protease trypsin (6.7 ng/µl) in NH_4HCO_3 (50 mM) buffer, as described by Campos et al. (2013) and Valerio et

al. (2014). Afterward the peptides were eluted directly onto the MALDI plate following the reversed-Phase ZipTip® C18 protocol (ZTC18S096, Millipore, Germany) for direct spotting on to a MALDI-TOF target plate. This was accomplished using the matrix α-cyano-4-hydroxycinnamic acid (5 mg/mL) prepared in acetonitrile (70%, v/v), TFA (0.1%, v/v), and ammonium phosphate (6 mM) to enhance peptide ionization.

5.2.7. Protein identification

Samples were analyzed using an Applied Biosystem 4800 Plus MALDI TOF/TOF Analyzer (AB SCIEX, Foster City, CA, USA) in MS and MS/MS mode according to Osorio and Reis (2013) . MALDI mass spectra were externally calibrated following the manufacturers instructions (TOF/TOF calibration mixture, AB SCIEX) and internal calibration was applied using trypsin autolysis peaks. Peptide mass spectra data was collected in positive ion reflector mode in the range of m/z 700-4000. Both MS and MS/MS spectra were analysed using the Mascot search engine (Version 2.4). The search included peaks with a signal-to- noise ratio greater than 10 and allowed for up to two missed trypsin cleavage sites, mass tolerance of 50 ppm, cysteine carbamidomethylation (fixed modification), methionine oxidation (variable modification), a charge state of +1o. MS/MS spectra were searched in a locally stored copy of the UniProt protein sequence database for the taxonomic selection *Danio rerio*. . For a match to be considered significant, protein scores with a probability greater than 95% ($p<0.05$), calculated by the Mascot software, were required.

5.3. Results and discussion

5.3.1. 2DE study and protein identification by MALDI-TOF mass spectrometry

Figure 13 shows an example of each 2DE Gel obtained from control zebrafish and irradiated groups (100 mGy, 500 mGy and 1000 mGy). A specific area showing high protein spot resolution (Figure 13) was selected for from the 2DE gels analysis. About 85 ± 21 protein spots were detected in this area of the 2DE gels, which were

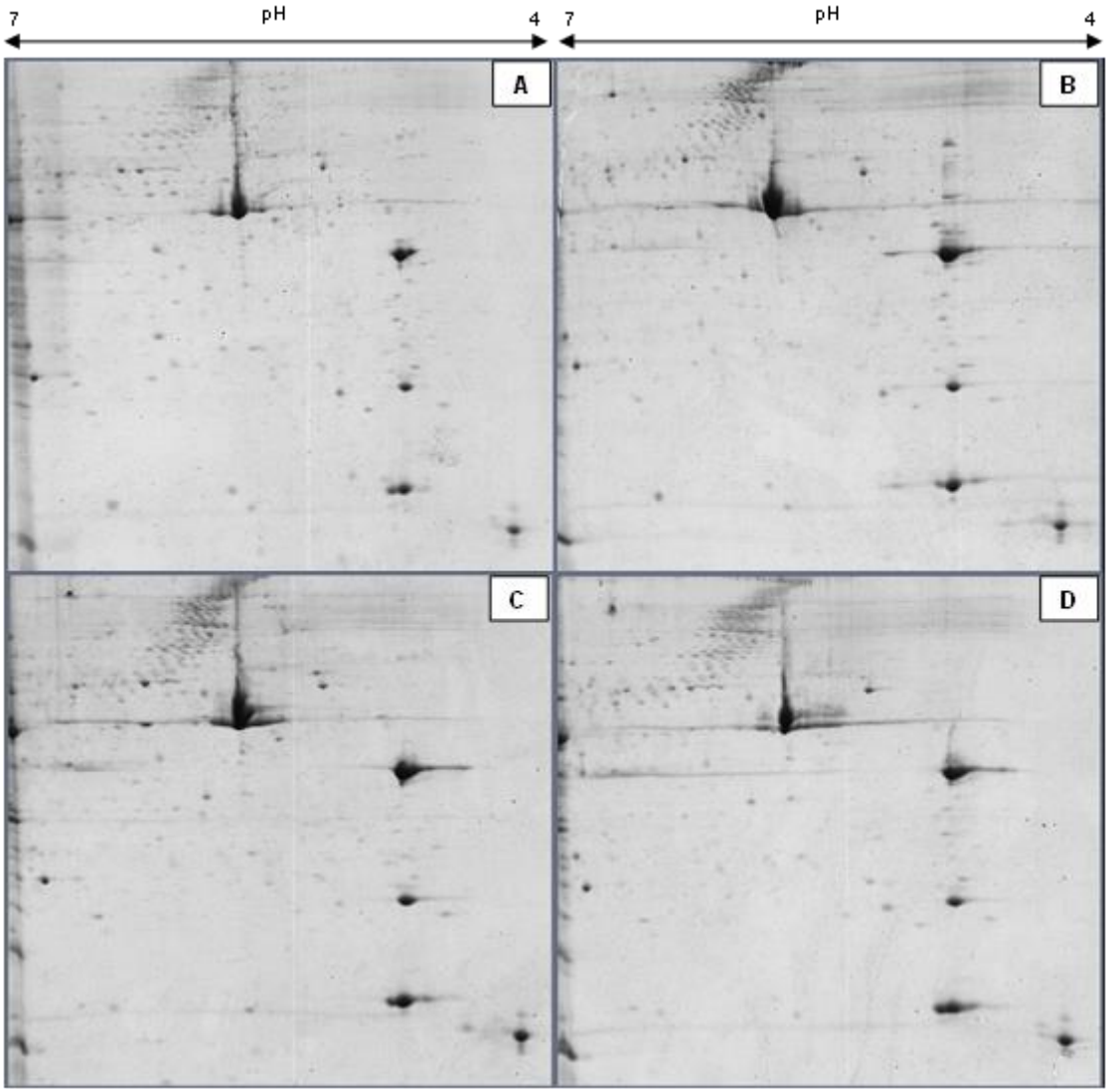

Figure 13 - Representative images of 2DGE Gels from muscle proteins of control zebrafish (A) and zebrafish exposed to 100 mGy (B), 500 mGy (C) and 1000 mGy (D) x-ray irradiation. Gels were loaded with 400 ug total muscle protein and separated along a 4-7 pH interval.

further analysed quantitatively. This group of proteins are distributed along the entire gel area, between the isoelectric points 4 and 7, and molecular masses 20 and 80 kDa (Figure 13). The statistical analysis led to the detection of a total of 32

differential protein spots in the treatment groups (27 protein spots were differentially expressed at 1000 mGy; 22 proteins at 500 mGy and 3 proteins at 100 mGy). The results thus point to an increase in proteome changes related to the radiation dose.

Proteins with expression differences were subsequently analysed by MALDI-TOF mass spectrometry for identification. Peptide mass fingerprint and tandem mass spectrometry data were used to search for homologous proteins in the UNIPROT database, using the search engine MASCOT (Matrixscience). This analysis resulted in the unambiguous identification of 15 proteins (**5.5. Supplementary material**, Supplementary Table 1). The relative abundances and putative functions of the identified proteins are presented in Table 7.

Table 7 - Protein abundance variations in zebrafish muscle individuals subjected to X-rays. Normalized intensity ± standard error of each identified protein in control and exposed fish. Significant decrease (↓) in protein abundance (P<0.05) comparatively to the control.

Spot reference	Protein name [1]	Experimental groups				Accession Number [2]	Specie	Functions [3]
		control	100 mGy	500 mGy	1000 mGy			
1502	Creatine kinase (ckm)	6894.6 ± 571.0	↓2459.9 ± 1447.9	↓811.4 ± 313.8	↓1388.3 ± 736.9	Q7T306	*Danio rerio*	P: phosphorylation; F: kinase activity; F: transferase activity
2315	Proteasome subunit beta type-4 (psmb4)	319.3 ± 64.2	161.0 ± 73.5	↓76.3 ± 34.1	↓81.2 ± 48.8	Q568F3	*Danio rerio*	P: proteolysis; F: threonine-type endopeptidase activity; C: proteasome complex; C: nucleus; C: cytoplasm
2402	Apolipoprotein A-Ib precursor (apoa1)	1431.6 ± 288.0	↓403.9 ± 187.5	↓505.5 ± 145.5	↓407.3 ± 150.7	A0A0R4IKF0	*Danio rerio*	P: lipid transport; P: lipoprotein metabolic process; F: lipid binding; C: extracellular region
3402	Creatine kinase M-type (ckm)	1284.1 ± 233.3	874.0 ± 448.5	↓132.9 ± 71.9	↓125.3 ± 64.2	A8E5L0	*Danio rerio*	P: phosphorylation; F: kinase activity; F: ATP binding; C: cytoplasm
3407	Alpha-cardiac actin (actc1)	621.6 ± 153.3	533.0 ± 252.0	↓94.6 ± 78.8	↓237.2 ± 168.7	Q9PTR4	*Danio rerio*	P: muscle contraction; P: skeletal muscle fiber development; F: structural constituent of cytoskeleton; F: protein binding; C: cytoplasm
3502	Alpha-cardiac actin (actc1)	2384.0 ± 717.4	1959.7 ± 1232.2	↓230.5 ± 126.3	↓202.3 ± 49.2	Q9PTR4	*Danio rerio*	P: muscle contraction; P: skeletal muscle fiber development; F: structural constituent of cytoskeleton; F: protein binding; C: cytoplasm
3512	Alpha-cardiac actin (actc1)	256.7 ± 27.7	254.7 ± 175.1	↓62.1 ± 53.7	↓0.0 ± 0.0	Q9PTR4	*Danio rerio*	P: muscle contraction; P: skeletal muscle fiber development; F: structural constituent of cytoskeleton; F: protein binding; C: cytoplasm
3903	Actin (acta1)	2932.7 ± 598.2	1963.9 ± 834.3	↓1040.7 ± 165.1	↓939.4 ± 185.3	Q6XNL8	*Danio rerio*	P: muscle contraction; P: skeletal muscle fiber development; F: structural constituent of cytoskeleton; F: protein binding; C: cytoplasm
6402	Myosin regulatory light chain 2 (mylpf)	611.0 ± 191.5	171.6 ± 139.1	171.9 ± 148.8	↓0.0 ± 0.0	O93409	*Danio rerio*	P: muscle contraction; P: regulation of cell shape; F: myosin heavy chain binding; C: cytosol; C: myosin II complex
7301	Atrial myosin light chain (MYL3)	5218.3 ± 828.5	4441.4 ± 1619.6	↓1543.6 ± 306.1	↓2079.7 ± 476.7	Q7ZUB0	*Danio rerio*	P: muscle contraction; P: actin filament-based movement; P: myofibril assembly; F: microfilament motor activity; F: actin binding; F: calmodulin binding; C: cytoplasm
7603	14-3-3 protein gamma-1 (ywhag)	319.6 ± 34.5	167.6 ± 86.9	↓61.0 ± 52.8	↓31.2 ± 27.0	E7F354	*Danio rerio*	P: regulation of signal transduction; P: negative regulation of protein kinase activity; P: regulation of synaptic plasticity; F: protein kinase C binding; F: protein kinase C inhibitor activity; C: cytoplasm

Spot reference	Protein name [1]	Experimental groups				Accession Number [2]	Specie	Functions [3]
		control	100 mGy	500 mGy	1000 mGy			
8201	Translationally-controlled tumor protein homolog (tpt1)	1812.5 ± 185.9	1077.4 ± 367.6	879.5 ± 393.1	↓632.9 ± 146.3	Q9DGK4	*Danio rerio*	P: cell differentiation; P: regulation of apoptotic process; F: calcium ion binding; F: microtubule binding; C: cytoplasm
8402	Proteasome subunit alpha type-5 (psma5)	1547.8 ± 304.1	1007.6 ± 334.6	↓402.8 ± 126.7	729.3 ± 365.2	Q6TGV6	*Danio rerio*	P: proteasome-mediated ubiquitin-dependent protein catabolic process; F: endopeptidase activity; C: proteasome complex; C:nucleos; C: cytoplasm
8501	Tropomyosin alpha-1 chain (tpm1)	1257.3 ± 313.3	750.2 ± 317.2	↓287.2 ± 120.4	493.1 ± 291.1	P13104	*Danio rerio*	P: movement of cell or subcellular component; P: regulation of muscle contraction ; P: cytoskeleton organization; F: actin binding; F: structural constituent of muscle; C: cytoplasm; C: cytoskeleton
8503	Tropomyosin alpha-4 chain isoform 2 (tpm4)	257.9 ± 65.2	65.5 ± 74.7	52.1 ± 45.1	↓0.0 ± 0.0	Q7T3F0	*Danio rerio*	P: movement of cell or subcellular component; P: regulation of muscle contraction ; P: cytoskeleton organization; F: actin binding; F: structural constituent of muscle; C: cytoplasm; C: cytoskeleton

[1] Protein name and in parenthesis the respective gene identifier

[2] Accession number of the protein in UNIPROT database

[3] Representative biological processes (P), molecular functions (F) and preferential cellular location (C), based on the attributed gene ontology terms

These alterations mainly comprise the decrease of protein expression that may be associated with catabolic processes (probably associated with the elimination of damaged proteins) and/or the inhibition of protein synthesis.

The results demonstrate that the exposure to X-ray leads to alterations in zebrafish muscle major structural proteins such as actin, myosin and tropomyosin, which also are responsible for the major contractile functions of the organ. Several proteoforms of these proteins were identified in 2DE gels, most showing decreased abundances upon X-ray exposure. Other quantitative alterations were observed in proteins with functions in the proteasome and ubiquitin-dependent protein catabolism (proteasome subunit alpha type-5, proteasome subunit beta); kinase dependent regulation of signal transduction (14-3-3 protein gamma-1 and creatine kinase); lipid transport (apolipoprotein A-Ib) and a protein with a putative function in cell differentiation and regulation of apoptosis (Translationally-controlled tumor protein) (Table 7).

The results thus point to an action of X-rays in the zebrafish skeletal muscle cells that could impair the function of the contractile apparatus. The putative alterations of the proteasome functions and signaling pathways in the muscle cells may also contribute to additional cellular stress and impairment of the skeletal muscle main functions. These protein abundances alterations may prejudice muscle function that might result for example in abnormal swimming of zebrafish.

The proteomic profiles observed in the control and the irradiated groups support the hypothesis of a dose-dependent response to ionizing radiation. Some other authors have studied the effects of ionizing radiation on proteins (Marchetti *et al.* 2006) in cell lines (Pluder *et al.* 2011; Baselet *et al.* 2017), animal models (Guipaud *et al.* 2007; Smith *et al.* 2007) and even in man (Skiöld *et al.* 2011; Guipaud 2013). Some of our identified proteins have already been recognised as candidate protein biodosimeters of human exposure to ionizing radiation by others authors namely: CKM and APOA1 were identified in mice (Chen *et al.* 2005); PSMB4 was identified in human TK6 lymphoblastoid cells (Tapio *et al.* 2005); TPM1 was identified in human serum (Menard *et al.* 2006); TPT1 was identified in mice (Zhang et al. 2003) and in human T-lymphocyte leukemia cells (Szkanderova et al. 2005).

However, to the best of our knowledge, this is the first study reporting the effects of radiation in the zebrafish proteome, which we hope can boost the use of this species as a model in radiobiology.

5.3.2 Functional interactions of differentially expressed proteins

The functional relations between differentially abundant proteins (Figure 14) were analyzed through the STRING database. The STRING database (http://string-db.org) aims to provide an assessment and integration of protein-protein interactions (Szklarczyk et al. 2015).

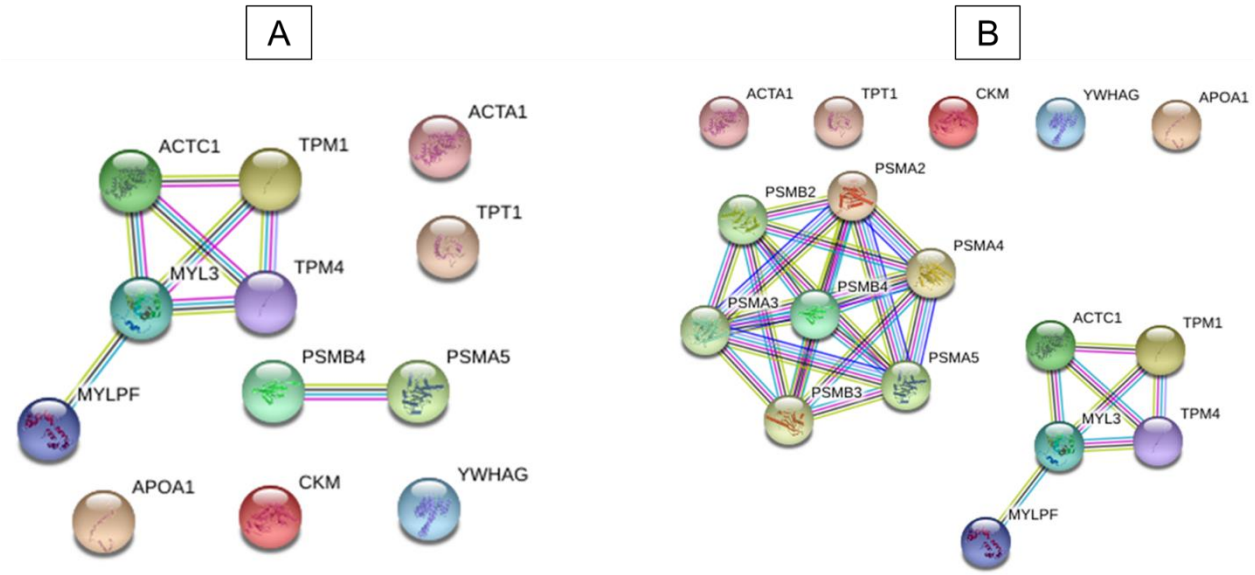

Figure 14 - Functional relationships and interaction network of differential muscle proteins from zebrafish exposed to 100 mGy, 500 mGy and 1000 mGy, provided by the software String (p=0.9). Interaction network with query proteins (A) and with 5 predicted interactors (B). Coloured lines distinguish the different data supporting the proposed interactions.

This analysis retrieved two important protein clusters that reflect their functional interrelationships. Significant interactions (p=0.9), based on protein experimental data or by interaction preditions, for the structural proteins (ACTC1, TPM1, TPM4, MYL3) and myosin regulatory light chain (MYLPF) form one of the clusters (Figure 14 A and B). This cluster evidences the greater effect of X-radiation on cytoskeletal elements which will impact on the structure of skeletal muscle cells and functions of this organ, as already suggested. On the other hand, the second cluster is formed by proteins PSMB4 and PSMA5 with functions at the level of the proteasome (Figure 14A) which suggests that X-rays also affect this metabolic process. This hypothesis of the proteosome as a molecular target is reinforced with

the finding that the PSMA5 and PSMB4 proteins are strongly associated with other constituents of the proteasome (Figure 14B), meaning that changes in these two proteins will very likely influence the functioning of the proteasome at several levels. For other differentially expressed proteins no functional associations have been established, however this result does not disregard the known functions of these proteins and their critical role in cell metabolism and therefore equally taken in consideration in the evaluation of the effects of radiation.

5.4. Conclusions

We confirm that 2DE and MALDI-TOF method are adequate analytical tools for study radiobiological effects in zebrafish. A large number of proteins were down-regulated suggesting that a remodelling of the muscle proteome occurs after the exposition to low doses of X-rays. Among the proteins identified are some involved in cytoskeleton structure, ubiquitin-dependent protein catabolism, kinase dependent regulation of signal transduction, lipid transport and a protein with a putative function in cell differentiation and regulation of apoptosis.

Future studies should be focused on the effects of even lower doses than those used in the present study and to include males and females aiming at detecting possible differences between genders.

5.5. Supplementary material

Supplementary Table 1 - Identification of protein spots showing differential abundance in zebrafish muscle individuals subjected to X-rays, by MALDI-TOF mass spectrometry.

Spot reference	Protein name	Accession Number	Matched Peptides[1]		Protein Score[2]	Sequences	Species
			MS	MS/ MS			
1502	Ckmb protein	Q7T306	11	6	392	K.GFTLPPHNSR.G K.ELFDPVISDR.H K.HLTDLNWENLK.G K.GGDDLDPNYVLSSR.V K.LSIEALNSLDGEFK.G K.TFLVWVNEEDHLR.V	Danio rerio
2315	proteasome subunit beta type-4	gi|62955575		3	127	K.AIHSWLTR.V R.EVIENKEEITKEEAR.E K.VNNSTILGASGDYADYQYLK.Q	Danio rerio
2402	apolipoprotein A-Ib precursor	gi|424036615		2	87	K.SALQVYADHLK.Q K.SLTHLDDTEFKDYK.V	Danio rerio
3402	creatine kinase M-type	gi|157787181		4	116	R.HGGYKPTDK.H K.GFTLPPHNSR.G K.ELFDPVISDR.H K.GGDDLDPNYVLSSR.V	Danio rerio
3407	alpha-cardiac actin	gi|6636384		3	143	R.GYSFVTTAER.E K.QEYDEAGPSIVHR.K K.SYELPDGQVITIGNER.F	Danio rerio
3502	alpha-cardiac actin	gi|6636384		5	226	R.DLTDYLMK.I R.GYSFVTTAER.E K.QEYDEAGPSIVHR.K K.SYELPDGQVITIGNER.F K.DLYANNVLSGGTTMYPGIADR.M	Danio rerio
3512	alpha-cardiac actin	gi|6636384		4	205	K.IIAPPERK.Y R.GYSFVTTAER.E K.QEYDEAGPSIVHR.K K.SYELPDGQVITIGNER.F	Danio rerio
3903	Actin	Q6XNL8	12	5	382	R.GYSFVTTAER.E K.QEYDEAGPSIVHR.K K.SYELPDGQVITIGNER.F R.VAPEEHPTLLTEAPLNPK.A K.DLYANNVLSGGTTMYPGIADR.M	Danio rerio
6402	myosin regulatory light chain 2	gi|18859049		12	552	K.NEELEAMIK.E K.VLDPEGTGSIK.K K.EAFTIIDQNR.D R.DVLASMGQLNVK.N K.GADPEDVIVSAFK.V K.NICYVITHGEEK.E K.LKGADPEDVIVSAFK.V K.EFLEELLTTQCDR.F K.NICYVITHGEEKEE.- K.NLWAAFPPDVAGNVDYK.N K.EASGPINFTVFLTMFGEK.L R.AAGGEGSSNVFSMFEQSQIQEYK.E	Danio rerio
7301	Atrial myosin light chain	Q7ZUB0	8	4	333	K.DAFQLFDR.T R.GTFEDFVEGLR.V K.ITFAQCGDLIR.A K.DRGTFEDFVEGLR.V	Danio rerio
7603	14-3-3 protein gamma-1	gi|125837336		3	126	R.YLAEVATGEK.R K.SYNEAHEISK.E K.SVTELNEALSNEER.N	Danio rerio
8201	Translationally-controlled tumor protein homolog	Q9DGK4	9	2	122	R.EDGVTPYMIFFK.D K.DIITGDEMFSDIYK.I	Danio rerio
8402	proteasome subunit alpha type-5	gi|45387823		2	47	R.GVNTFSPEGR.L R.LFQVEYAIEAIK.L	Danio rerio
8501	tropomyosin alpha-1 chain	gi|18859505		3	103	R.IQLVEEELDR.A R.KLVIVEGELER.T K.KATDAEGDVASLNR.R	Danio rerio
8503	tropomyosin alpha-4 chain isoform 2	gi|47085929		2	59	R.KLVILEGELER.A K.YSEKEDKYEEEIK.V	Danio rerio

[1] Number of peptides matched and fragmented peptides in MALDI-TOF/TOF

[2] Significant identification scores obtained with the Mowse algorithm (P<0.05)

Chapter 6

Overall Conclusions and Final Remarks

Over the last decades, zebrafish has emerged as a major animal model in many fields of biomedical research due to a number of well-known practical advantages and the substantial homology between its genome and the human genome. In spite of this, the use of zebrafish in radiobiology is still scarce and most often restricted to the embryonic stage. The present work aimed at exploring the potential of adult zebrafish as a model for radiobiological studies, particularly in evaluating the effects of low-dose ionizing radiation.

Using a simple and easily reproducible irradiation protocol, adult zebrafish were exposed to acute irradiation by X-ray from a radiotherapy equipment, at a low-dose range for medical use (100-1000 mGy). Effects were evaluated at the cellular level (DNA damage in blood cells), the biochemical level (antioxidant enzyme activity and lipid peroxidation in brain and liver) and the molecular level (protein expression in muscle). Our results showed that:

- DNA damage in blood cells of irradiated adult zebrafish increased in a dose dependent manner at one day post-irradiation, but thereafter DNA repair should have occurred since the level of damage returned to that of non-irradiated fish. In offspring from irradiated adults, the level of DNA damage in whole-body cells was directly correlated with the radiation dose of parental exposure. This indicates that DNA damage induced in parents were transmitted to the progeny, and confirms the potential of zebrafish for studies on transgenerational effects of low-dose ionizing radiation. Overall results also confirm the comet assay as a reliable and sensitive technique for measuring radiation-induced DNA damage both in blood samples of adult zebrafish and in whole-body larvae.

- The oxidative damage (lipid peroxidation level) caused by radiation and the antioxidant response (catalase activity) elicited by radiation followed different patterns over time post-irradiation depending on the gender (male vs female), the organ (brain vs liver) or the dose (lower vs higher) under consideration. Data point to possible hyper-radiosensitivity to low-dose exposures (≤500 mGy) in males and females. However, male liver appears to show wider range of hypersensitivity as well as possible increased

susceptibility to the bystander effect. Future studies should be targeted at identifying other antioxidant and oxidative stress biomarkers involved in the response to low-dose ionizing radiation.

- A large number of muscle proteins were down-regulated in irradiated fish compared to non-irradiated fish, as revealed by the 2DE, suggesting a remodelling of the muscle proteome after the exposition to low doses of X-rays. Moreover, differences in the proteomic profile among groups suggest a dose-dependent response to ionizing radiation. The proteins differentially abundant are involved in cytoskeleton structure, ubiquitin-dependent protein catabolism, kinase dependent regulation of signal transduction, lipid transport and a protein with a putative function in cell differentiation and regulation of apoptosis.

Overall results demonstrate that adult zebrafish should be considered as a valuable model in radiobiology, particularly for studying effects of low-dose ionizing radiation.

Chapter 7

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