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## ***In Vivo* Space Radiation-Induced Non-Targeted Responses: Late Effects On Molecular Signaling In Mitochondria**

Mohit R. Jain<sup>1</sup>, Min Li<sup>2</sup>, Wei Chen<sup>1</sup>, Tong Liu<sup>1</sup>, Sonia M. de Toledo<sup>2</sup>, Badri N. Pandey<sup>2</sup>, Hong Li<sup>1</sup>, Bernard M. Rabin<sup>3</sup>, and Edouard I. Azzam<sup>\*,2</sup>

<sup>1</sup>Department of Biochemistry and Molecular Biology, University of Medicine and Dentistry of New Jersey, New Jersey Medical School Cancer Center, Newark, New Jersey, USA

<sup>2</sup>Department of Radiology, University of Medicine and Dentistry of New Jersey, New Jersey Medical School Cancer Center, Newark, New Jersey, USA

<sup>3</sup>Department of Psychology, University of Maryland Baltimore County, Baltimore, MD, USA

### **Abstract**

The lack of clear knowledge about space radiation-induced biological effects has been singled out as the most important factor limiting the prediction of radiation risk associated with human space exploration. The expression of space radiation-induced non-targeted effects is thought to impact our understanding of the health risks associated with exposure to low fluences of particulate radiation encountered by astronauts during prolonged space travel. Following a brief review of radiation-induced bystander effects and the growing literature for the involvement of oxidative metabolism in their expression, we show novel data on the induction of *in vivo* non-targeted effects following exposure to 1100 MeV/nucleon titanium ions. Analyses of proteins by two-dimensional gel electrophoresis in non-targeted liver of cranially-irradiated Sprague Dawley rats revealed that the levels of key proteins involved in mitochondrial fatty acid metabolism are decreased. In contrast, those of proteins involved in various cellular defense mechanisms, including antioxidation, were increased. These data contribute to our understanding of the mechanisms underlying the biological responses to space radiation, and support the involvement of mitochondrial processes in the expression of radiation induced non-targeted effects. Significantly, they reveal the cross-talk between propagated stressful effects and induced adaptive responses. Together, with the accumulating data in the field, our results may help reduce the uncertainty in the assessment of the health risks to astronauts. They further demonstrate that 'network analyses' is an effective tool towards characterizing the signaling pathways that mediate the long-term biological effects of space radiation.

### **Keywords**

High charge and high energy particles; ionizing radiation; liver; mitochondria; non-targeted responses; oxidative metabolism; space radiation

### **INTRODUCTION**

During radiotherapy, accidents, or unavoidable exposures in the course of specialized activities, it is more likely that an individual will be partially rather than wholly exposed to ionizing radiation. However, in early studies of the biological effects of radiation, it has been

\*Address for correspondence to this author at Department of Radiology, University of Medicine and Dentistry of New Jersey, New Jersey Medical School Cancer Center, 205 South Orange Avenue, Room - F1212, Newark, NJ 07103, USA; Tel: +1-973-972-5323; Fax: +1-973-972-1865; azzamei@umdnj.edu.

widely accepted that to elicit an important biological response, the energy from the radiation must be deposited in the nucleus of the targeted cell [1]; that is, bystander cells that neighbor irradiated cells would be unaffected. However, as described here and in the accompanying manuscripts in this issue of the journal [2-6], observations made over the last two decades have clearly indicated that the biological effects of ionizing radiation are not confined to the cells that have been directly targeted by the radiation, but involve a number of non-targeted and delayed effects [7-13]. Whereas intercellular communication of signaling molecules that lead to cell death among irradiated tumor cells, and between irradiated and bystander tumor cells would be beneficial, the transmission of stressful effects from irradiated tumor or normal cells to other normal bystander cells, and the persistence of such effects in their progeny, would have profound implications for long term health risks. Hence, the study of non-targeted effects of ionizing radiation is not only significant to our understanding of intercellular communication, but is also relevant to both clinical and radioprotection issues [2, 3, 5].

### The Ionizing Radiation-Induced Bystander Effect

The ionizing radiation-induced bystander effect has been broadly defined as the occurrence of biological effects in unirradiated cells as a result of exposure of other cells to radiation [14]. Bystander effects have been extensively observed in cell cultures exposed to low fluences of  $\alpha$  particles that target only a small fraction of the cells in the population [15]. They were also detected in co-cultures of bystander cells and cells exposed to low linear energy transfer (LET) radiations [16-20] or to high LET radiations besides  $\alpha$ -particles [21-23], thus highlighting their relevance to radiotherapy, diagnostic radiology, and risk of environmental and occupational exposures [10-12]. Upregulation of stress-responsive proteins, genetic and epigenetic changes, induction of cell cycle checkpoints and cell killing were shown to occur in both irradiated and neighboring bystander cells [2, 3, 6, 8, 10]. The effects occur in various cell types of human and rodent origin at different stages of growth [2, 9, 12, 24-26]. Mechanistic studies have shown that direct and indirect modes of intercellular communication and oxidative metabolism can mediate these effects [15, 27, 28]; however, the exact molecular steps involved have not been defined [2, 15]. Thus, quantitative and qualitative understanding of these biological effects through system biology approaches should lead to better estimation of health risks [5, 29-31].

In addition to observations in tissue culture, significant radiation-induced bystander effects were detected in 3-dimensional human tissue models [32-34], and in animal experiments [6, 35-37]. With particular relevance to cancer risk, non-targeted oncogenic radiation effects were observed in the cerebellum of radiosensitive mice, when only the rest of their body was X-irradiated [38]. The occurrence of such *in vivo* stressful non-targeted effects would have significant consequences during particular activities, such as mining or space travel, when often only parts of the human body are irradiated at any one time [39]. In case of deep space travel, it has been estimated that an astronaut's body would be exposed, *daily*, to very low mean doses of densely ionizing radiations [40]. At the typical doses encountered [41, 42], only a very small fraction of cells in the human body would experience the large ionization events created along the tracks of such radiations [41, 42]. Moreover, the radiation traversals would be separated in tissue location and time [22].

### Non-Targeted Effects and Space Radiation

The health effects of concern from space radiation are those caused by low dose-rate protons and energetic heavy ions for which there is a paucity of data. Due to their high biological effectiveness, high charge and high energy (HZE) particles produce a significant fraction of the effective dose received during missions in space. Whereas photons deposit their energy in a rather diffuse pattern, energy deposition by high-LET energetic particles is structured

along linear tracks and is far less homogeneous than low LET radiation [43-45]. HZE particles can travel substantial distances in tissue and an enhanced rate of energy loss occurs at the end of their range where high LET values in tissue can be reached (Bragg peak) [46]. Thus, HZE particles likely result in unique biological responses [46-48]. The lack of clear knowledge about these responses, including the induced non-targeted effects [49-51], has been singled out by the US National Academies [52] and recent studies [39] as the most important factor limiting the prediction of radiation risk associated with human space exploration.

During prolonged space travel, astronauts are exposed to ionizing radiation from a variety of sources including galactic cosmic rays, solar protons, and high energy electrons and protons trapped by the earth's magnetic field (Van Allen Belts). Galactic cosmic rays consist mainly of protons, but also include a small component of HZE particles, which due to their high biological effectiveness, produce a significant fraction of the effective dose received during missions in space (reviewed in [22]). Shielding by the earth's thick atmosphere and its magnetic fields provides some protection to astronauts traveling in low earth orbit (e.g. space shuttle and international space station). However, this shielding is lost in deeper interplanetary space and at the Martian surface. Cucinotta *et al.* [42] calculated that during a mission to Mars, every cell nucleus in an astronaut's body would be hit by a proton or a secondary electron every few days, and by an HZE particle about once a month.

When monolayer cell cultures are exposed to low mean doses from HZE particles, a significant fraction of the cells will not be traversed by a high-LET radiation track [43]. However, some of these cells are irradiated by  $\delta$ -rays. A 1 GeV/nucleon  $^{56}\text{Fe}$  ion generates  $\delta$ -rays with ranges up to 270  $\mu\text{m}$  [53]. The cells traversed by HZE particles may also impart stressful bystander effects on nearby cells [51, 54, 55]. However, the study of *in vivo* non-targeted effects is only emerging [36-38, 56], in particular in the context of space radiation. The occurrence of delayed stressful effects in targeted and/or non-targeted tissues of animals exposed to particulate radiations found in deep space would have significant relevance to space exploration. Here, we investigate biochemical changes in mitochondria of non-targeted liver of rats that were exposed to energetic titanium ions (1100 MeV/nucleon) delivered to the cranial region 20 months earlier. We focused on examining biochemical changes in mitochondria as our earlier tissue culture studies have shown that the functions of these organelles are highly sensitive to ionizing radiation exposures [57, 58], and oxidative metabolism mediates ionizing radiation-induced bystander effects [59-64].

### Mitochondria, Oxidative Metabolism and Bystander Effects

Mitochondria are the organelles that mediate respiration and ATP synthesis in eukaryotic cells. They consume about 90% of the body's oxygen and participate in numerous indispensable metabolic pathways (e.g. synthesis of heme, nucleotides, lipids, and amino acids) and mediate intracellular homeostasis of inorganic ions [65]. Mitochondrial function is, therefore, essential for the viability of eukaryotic cells.

Mitochondria have inner and outer membranes, a soluble matrix, and an intermembrane space (see scheme in Fig. (1)), and new mitochondria are formed by the growth and division of preexisting organelles. Each cell has many mitochondria, and the amount of mitochondria in the cell is dependent on the particular cell's function. Mitochondria localize in regions where the ATP demand is high; thus, mitochondrial composition varies in different tissues and cell types [66].

The mitochondria's role as "powerhouse of the cell" is fulfilled through aerobic cellular respiration. It is the cell's principal source of energy, and includes the tricarboxylic acid (TCA) cycle, the electron transport chain (ETC) that is typically composed of five multi-

subunit enzymes (complexes I-V), and oxidative phosphorylation. Each of these processes takes place in different regions of the organelle. The TCA cycle occurs in the mitochondrial matrix, and oxidative metabolism of carbohydrates, lipids, and amino acids integrate into the TCA cycle to produce NADH and FADH<sub>2</sub>. These essential intermediates are electron-rich donors that enter the ETC on the inner mitochondrial membrane for use in ATP production [67]. Ionizing radiation is energetic and penetrating, and may disrupt the formation/maintenance of some of the macromolecular respiratory complexes, which may be revealed by biochemical approaches, including proteomic approaches. Detailed characterization of persistent changes in the protein composition of mitochondria in irradiated or bystander cells and in their progeny is only emerging. Altered protein expression in mitochondria may affect reactive species level and the overall redox environment of the cell.

A consequence of energy production through oxidative metabolism is the production of reactive oxygen species (ROS) byproducts. The premature leakage of electrons, mainly from complex I and complex III, and complex II to a minor extent, results in the reduction of O<sub>2</sub> to create superoxide (O<sub>2</sub><sup>-</sup>) [68, 69]. Apart from this normal basal level of ROS production, radiation damage to mitochondria, the hub of oxidative metabolism, causes further leakage of electrons from the ETC, and therefore results in excess O<sub>2</sub><sup>-</sup> generation [70]. This occurs in addition to O<sub>2</sub><sup>-</sup> and other chemical species produced during cellular water radiolysis at the time of irradiation, which together results in enduring perturbation of oxidation/reduction reactions that contribute to maintenance of the cellular redox environment [70, 71]. Thus, though a burst of excess ROS is initially produced at the time of irradiation and is believed to persist for only microseconds or less [72], radiation-induced oxidative stress on the cell may be prolonged due to persistent long-term effects on mitochondrial functions, primarily oxidative metabolism [70], which is likely to involve changes in protein expression in mitochondria.

Short- and long-term radiation-induced cellular oxidative stress could result in damage to mitochondrial DNA (mtDNA) and/or nuclear DNA (nuDNA) coding for mitochondrial ETC subunits as well as the biochemical machinery necessary for the proper expression and assembly of ETC proteins. In fact this possibility has been previously proposed for metabolic oxidative damage that occurs during the aging process [73, 74]. Compared with nuDNA, mtDNA contains limited repair capability [75, 76]. Therefore, subsequent to radiation exposure, mtDNA might be preferentially damaged or lost due to oxidative stress with an ensuing decrease in respiratory chain activity and loss of mitochondrial function. Mutations in mtDNA causing disruptions in the proper assembly and/or function of mitochondrial ETCs could lead to an increase in residence time and/or accessibility of reduced components of the ETCs to O<sub>2</sub> resulting in an increase in the probability of prooxidant formation [70]. The net consequence being a condition of persistent metabolic oxidative stress that could continue to cause further oxidative damage to critical biological structures long after radiation exposure. Significantly, radiation-induced damage to mtDNA in directly targeted or bystander cells could become a heritable trait and contribute to radiation-induced genomic instability [77]. Insertion of mtDNA in nuDNA may affect the structure of nuclear genome and result in permanent changes in gene expression.

Whereas persistent excess in ROS level in irradiated cells may result from damage to components of the mitochondrial respiratory processes and an effect on plasma membrane oxidases [78, 79], it may also result from a disruption in antioxidant enzyme activity. A decrease in the availability of antioxidants or a limited accessibility of the antioxidants to the site of radiation-induced free radical production and oxidative damage contributes to significant damage to cellular macromolecules, including DNA, RNA, proteins, and lipids, which may further contribute to genomic instability, cancer, accelerated aging and the development of degenerative diseases [80].

Though most studies have investigated changes in mitochondria functions in irradiated cells [57], here, we examine persistent changes in protein levels in mitochondria of non-targeted liver of Sprague-Dawley rats that were cranially-exposed 20 months earlier to energetic titanium ions (1100 MeV/nucleon). The goal is to investigate changes in proteins associated with metabolic pathways, with the aim of gaining greater understanding of the mechanisms underlying long-term space radiation health risks.

## MATERIALS AND METHODS

Eight-weeks-old male Sprague-Dawley rats were exposed to 0 or 50 cGy from 1100 MeV/nucleon titanium ions (mean LET ~ 106 keV/ $\mu\text{m}$ ) at the NASA Space Radiation Laboratory located at the Brookhaven National Laboratory in Upton, NY, USA. Description of the facility and radiation beam information can be found at <http://www.bnl.gov/medical/nasa/LTSF.asp>. The rats were housed in AAALAC-accredited facilities and were maintained on a 12-h light/dark cycle in a temperature and humidity controlled room. Food and water were continuously available, and the research protocol was approved by the Institutional Animal Care and Use Committees of all the institutions implicated in the study.

During the irradiation, the rats were placed in well-ventilated plastic restraining tubes that restricted the movement of the animal. The plastic tube was placed perpendicular to the beam and positioned so that the head of the rats was in the center of the beam. As such, the rat's shoulders may have also received some radiation dose. The rats were sacrificed by CO<sub>2</sub> asphyxiation 20 months after exposure, liver tissue was harvested, rinsed in buffered Minimal Essential Medium (pH 7.4). Liver from three individual rats were pooled from each experimental group and mitochondria was harvested as described [81, 82]. The mitochondrial proteins were isolated [57]; they were purified using Biorad's ReadyPrep 2-D cleanup kit (Catalog # 163-2130), and subjected to 2-dimensional gel electrophoresis. Briefly, 100  $\mu\text{g}$  of proteins in a total of 185  $\mu\text{L}$  of the rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 0.2% Biolytes (pH 5-8), 0.01% Bromophenol Blue and protease inhibitor) was applied to 11 cm Bio-Rad ReadyStrip IPG Strips (pH 5-8) for overnight rehydration. First-dimension isoelectric focusing was performed on a Biorad PROTEAN IEF System at the UMDNJ Center for Advanced Proteomics Research (<http://njms.umdnj.edu/proweb>), as described by the manufacturer for a total focusing time of 75,000 Vhrs. The strips were equilibrated with an equilibration solution I (6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2% (w/v) DTT) for 15 min; they were then further equilibrated with an equilibration solution II (containing 6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2.5% (w/v) iodoacetamide) for 15 min and directly applied to a 12.5% isocratic SDS-PAGE gel for second dimensional electrophoresis. The resulting gel was then fixed in fixing solution (10% acetic acid and 40% ethanol) for 30 min and stained overnight with SYPRO Ruby (Bio-Rad). The gels were destained with destaining solution (10% methanol, 7.5 % acetic acid) for 2 h. After washing with water, they were scanned on a 9400 Typhoon Variable Mode Imager (GE Healthcare) using a green laser (532 nm) and 610BP30 emission filter. Protein quantitation on SYPRO ruby stained gels was performed by PDQuest 2-D Analysis Software (Bio-Rad) and protein spots whose expression levels significantly changed were cut for mass spectrometric (MS) identification.

### Mass Spectrometry and Protein Identification

Protein spots from the SYPRO Ruby stained 2-D gel were picked for protein identification. The gel bands were diced into 1 mm<sup>3</sup> pieces and washed with 30% acetonitrile (ACN) in 50 mM ammonium bicarbonate prior to DTT reduction and iodoacetamide alkylation. Trypsin was used for digestion at 37°C overnight. The resulting peptides were extracted with 30  $\mu\text{L}$

of 1% trifluoroacetic acid (TFA) followed by C<sub>18</sub> ZipTip desalting. For the MS analysis, the peptides were mixed with 7 mg/ml  $\alpha$ -cyano-4-hydroxy-cinnamic acid matrix (in 60% CAN) in a 1:1 ratio and spotted onto a matrix assisted laser desorption/ionization (MALDI) plate. The peptides were analyzed on a 4800 MALDI TOF/TOF analyzer (Applied Biosystem, Framingham, MA). Mass spectra ( $m/z$  880-3,200) were acquired in positive ion reflector mode. Fifteen most intense ions were selected for subsequent MS/MS sequencing analysis in 1 kV mode. Protein identification was performed by searching the combined MS and MS/MS spectra against the NCBI rat sequence database (<ftp://ftp.ncbi.nih.gov/blast/db/FASTA>) using a local MASCOT search engine (V. 1.9) on a GPS (V. 3.5, ABI) server. Protein containing at least two peptides with Confidence Interval (C. I.) values of no less than 95% was considered being identified.

## RESULTS AND DISCUSSION

The transfer of radiation energy to living tissues causes ionization of atoms and molecules and breaks chemical bonds, which initiates a series of biochemical and molecular signaling events that culminate in transient or permanent physiological changes [83]. In evaluating these changes, it had been traditionally thought that the important biological effects were confined to the targeted cells, and were a result of the direct interaction of the radiation with the DNA of these cells [8, 9]. It was generally presumed that no effect would be expected in cells in the population that receive no direct radiation exposure. However, since the landmark paper by Nagasawa and Little [14], the evidence for the existence of non-targeted/bystander effects of radiation has accumulated and has been observed in many laboratories (reviewed in [11, 84, 85] and in [2, 3] in this issue). Whereas, radiation-induced bystander effects have been well characterized *in vitro*, their expression *in vivo* is less clear, particularly in the context of space radiation. Our ongoing studies (not shown) with rodents that received partial body irradiation from HZE particles such as those found in deep space have clearly shown that mitochondrial functions are impaired in different non-targeted tissues. For example, import of proteins that localize in the mitochondrial matrix was greatly reduced in non-targeted liver of cranially-irradiated rats. The effect was coupled with extensive and persistent protein carbonylation. To gain understanding of the mechanisms underlying these changes, we examined alterations in protein levels in mitochondria of liver of rats that were exposed to 50 cGy from <sup>48</sup>Ti (1100 MeV/nucleon, mean LET ~ 106 keV/ $\mu$ m) targeted to the cranial region.

Two-dimensional gel analyses revealed that the level of several proteins was significantly altered as shown in Table (1). In particular, acetyl-Coenzyme A acyltransferase 2, hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit were found to be down-regulated by at least 2.5 fold. All these proteins are involved in fatty acid elongation in mitochondria (Fig. (2)). Fatty acid synthesis in mitochondria occurs by an acetyl-CoA-dependent elongation of fatty acids [86-88]. The pathway involves elongation of either palmitate or other dietary fatty acids to give rise to longer fatty acids. Fatty acid elongation is important to store energy and to synthesize lipids that are important for cellular functions, including membrane components [89]. These findings suggest that irradiation results in deficiency in the ability of certain non-targeted tissues to synthesize and store fatty acids. Our ongoing studies are investigating whether this deficiency occurs also in the irradiated or other non-irradiated tissues, and whether protein degradation is involved in the reduced expression levels.

Further, relative to control, the expression level of DNAJC3, DnaJ (Hsp40) homolog, subfamily C, member 3, also known as protein kinase inhibitor p58, was increased by 1.6 fold in the non-targeted liver of the irradiated rats (Table (1)). DNAJC3 is involved in the

endoplasmic reticulum stress response. In particular, it is also a target of Nrf2, a critical transcription factor for mediating amplification of the mammalian defense system against environmental stresses (Fig. (3)). The Nrf2 protein is known to be a key regulator of the response to oxidative stress [90, 91]. It acts as a nuclear transcription factor that controls the expression and coordinated induction of a battery of defensive genes encoding detoxifying enzymes and antioxidant proteins. Thus it represents a mechanism of critical importance for cellular protection and cell survival [92]. Similarly, the expression level of carboxylesterase 1 (monocyte/macrophage serine esterase 1) was found to be increased by 3.6 fold. Carboxylesterase 1 plays a role in detoxification in the lung and/or protection of the central nervous system from ester or amide compounds [93], and carboxylesterase deficiency may be associated with non-Hodgkin lymphoma or B-cell lymphocytic leukemia [94]. Furthermore, carboxylesterase 1 is a target of pregnane X receptor (PXR) [95], a key regulator of the body's adaptive defense mechanism against toxic substances including foreign chemicals (xenobiotics). Investigation of the inter-relationship between adaptive and bystander responses has been proposed as critical to understanding the health risks of low-level radiation exposures [85, 96]. Together, our findings provide novel mechanistic evidence for the cross-talk between these phenomena and show that although stressful effects may be propagated from irradiated to non-irradiated tissues, key pathways are activated to cope with the induced stress. Significantly, in terms of evaluating the health risks of exposure to space radiation, our results indicate that these changes persist and are observed several months after the irradiation.

## CONCLUSIONS

Our results support the concept that ionizing radiation interferes with cellular functions at all levels of the organism's organization, including the targeted and non-targeted tissues. They challenge the traditional paradigm that the important biological effects of ionizing radiation are a result of DNA damage by its direct interaction with the nucleus [8]. They indicate that metabolism is implicated in radiation-induced non-targeted effects. However, clear evidence explaining how these events occur is still lacking.

Our data show that ionizing radiation induces alterations, by non-targeted effects, in the protein composition of mitochondria, the organelles where most reactions involved in oxidative metabolism occur. The analyses of changes in protein levels in mitochondria of non-targeted liver of rats that received partial body irradiation from energetic titanium ions has shown regulation of proteins involved in signaling events that mediate both stressful non-targeted effects and protective adaptive responses. Significantly, the data cement the concept that *in vivo* space radiation-induced non-targeted effects do indeed exist [39, 97]. Importantly, they show that these effects persist and are observed long after the exposure (20 months). They reveal the cross-talk between late non-targeted stressful effects (Fig. (2)) and protective adaptive responses (Fig. (3)).

Gene expression is regulated at multiple levels [6], and elucidating the molecular events leading to the altered expression of the proteins investigated in this study (Table (1)) should lead to greater understanding of the mechanisms underlying the long-term effects of space radiation on health and disease. The available data bases on long-term effects on astronauts' health are likely to remain extremely limited in the near future; therefore, understanding of the mechanisms underlying the biological responses to energetic proton- and heavy ion-exposures will help reduce the uncertainty in the assessment of the health risks to astronauts (and public travelers in the future) from low-level exposure to space radiation [98]. Expansion of these studies to early and late time points after exposure to acute and protracted particulate space radiations should increase our understanding of the signaling pathways involved. It may also guide the formulation of interventional strategies, including

dietary antioxidants, substrates for the regeneration of reducing equivalents necessary for antioxidant pathways and genetic manipulations [98-102]. Finally, our study shows that network analyses is indeed an effective tool towards characterizing the signaling pathways that mediate the biological effects of space radiation.

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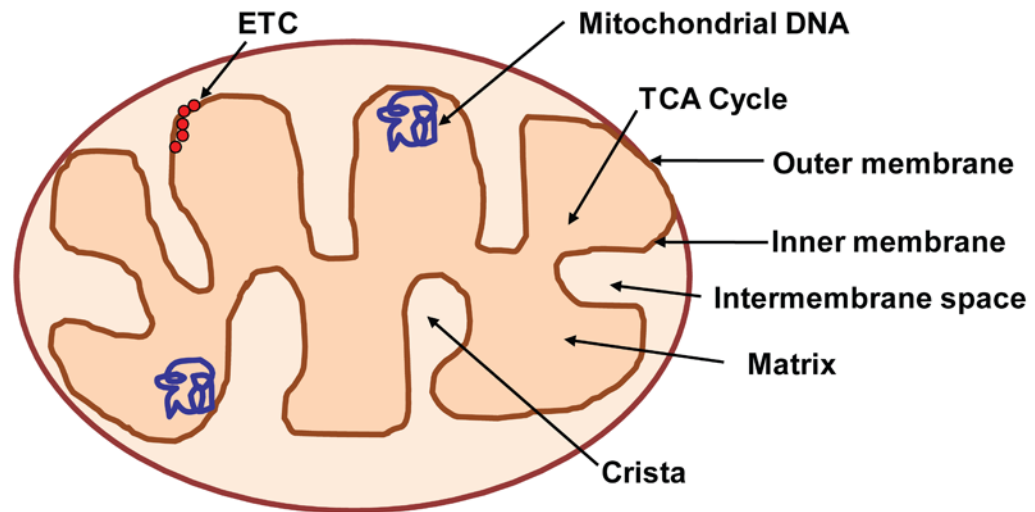
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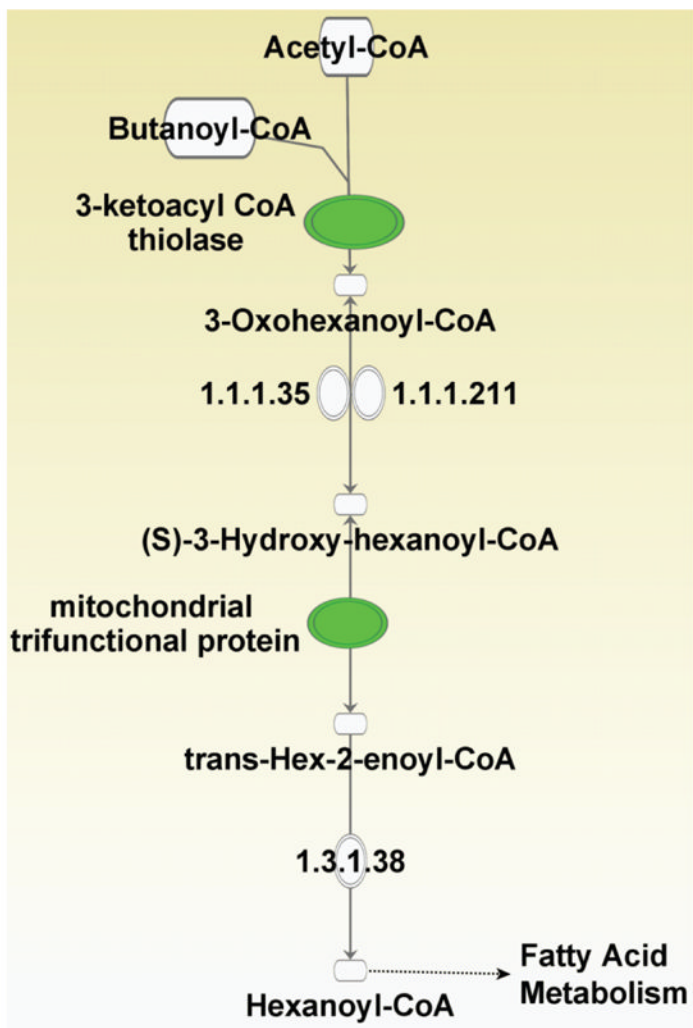
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## ABBREVIATIONS

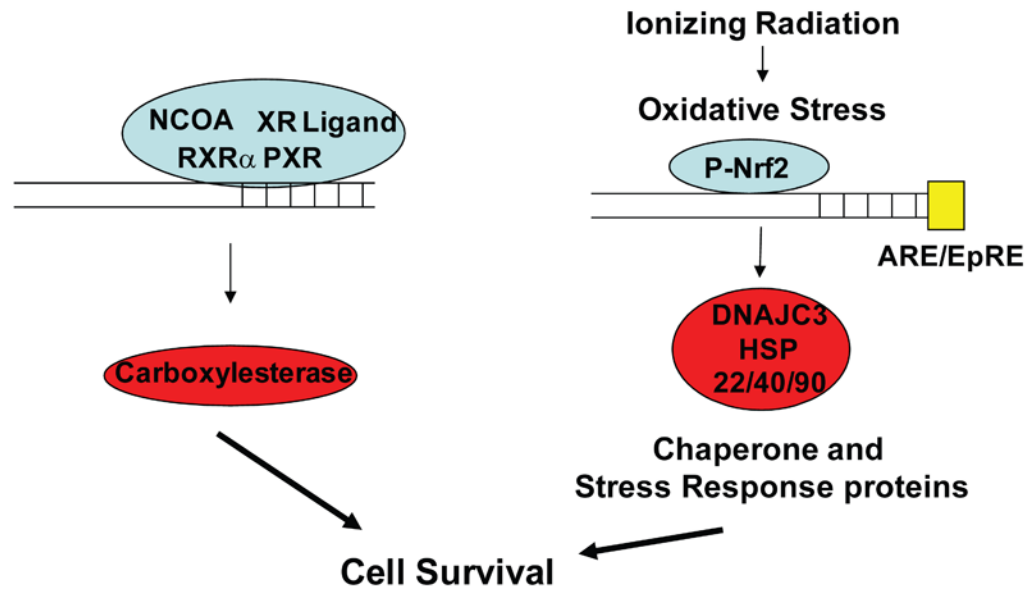
<b>2-D</b>	Two-dimensional
<b>AAALAC</b>	Association for assessment and accreditation of laboratory animal care international
<b>ACN</b>	Acetonitrile
<b>ATP</b>	Adenosine triphosphate
<b>CHAPS</b>	3[(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid
<b>CoA</b>	Coenzyme A
<b>DNAJC3</b>	DnaJ (Hsp40) homolog, subfamily C, member 3
<b>DTT</b>	Dithiothreitol
<b>ETC</b>	Electron transport chain
<b>FADH<sub>2</sub></b>	Flavin adenine dinucleotide (reduced form)
<b>HZE</b>	High charge and high energy
<b>IEF</b>	Isoelectric focusing
<b>IPG</b>	Immobilized pH gradient
<b>LET</b>	Linear energy transfer
<b>MALDI</b>	Matrix assisted laser desorption/ionization
<b>MASCOT</b>	Modular approach to software construction operation and test
<b>MS</b>	Mass spectrometry
<b>mtDNA</b>	Mitochondrial DNA
<b>NADH</b>	Nicotinamide adenine dinucleotide (reduced form)
<b>NCBI</b>	National center for biotechnology information
<b>Nrf2</b>	Nuclear factor (erythroid-derived 2)-like 2
<b>nuDNA</b>	Nuclear DNA
<b>ROS</b>	Reactive oxygen species
<b>SDS-PAGE</b>	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
<b>TCA</b>	Tricarboxylic acid cycle
<b>TOF</b>	Time of flight



**Fig. (1).**  
A simplified schematic of the structure of the mitochondrion



**Fig. (2).** Proteins down-regulated in mitochondria of non-targeted liver of rats whose head was exposed 20 months earlier to 1100 MeV/nucleon titanium ions participate in fatty acid elongation in mitochondria. (The down-regulated proteins (highlighted in green) are 3-ketoacyl CoA thiolase and mitochondrial trifunctional protein alpha subunit).



**Fig. (3).**

Up-regulated carboxylesterase and DNAJC3 (heat shock proteins) in mitochondria of non-targeted liver of rats whose head was exposed 20 months earlier to 1100 MeV/nucleon titanium ions participate in cell survival functions (NCOA: Nuclear receptor coactivator 1; RXR $\alpha$ : Retinoid receptor  $\alpha$ ; PXR: Pregnane X receptor; Nrf2: NF-E2-related factor 2; ARE/EpRE: Antioxidant response element/Electrophile response element; DNAJC3: DnaJ homolog subfamily C member 3)



**Table 1**

List of proteins found altered in non-targeted liver of rats whose head was exposed to 50 cGy from  $^{48}\text{Ti}$  ions (1100 MeV/nucleon)

Protein Name	Accession No.*	Ratio (Non-Targeted /Control)
3-ketoacyl-coA thiolase, mitochondrial	18426866	-2.37
Alpha-1-antiproteinase	112889	-2.57
Ba1-647	33086640	-2.17
Carbamoyl-phosphate synthetase 1 precursor	8393186	-2.57
Carboxylesterase 3 precursor	140969642	3.67
Cathepsin D	42476045	-3.7
Choline dehydrogenase	1154950	-2.17
Fibrinogen gamma chain	61098186	-1.57
Ganionic trypsin-1 precursor	6981420	-2.17
Glycerate kinase	157821525	-2.57
Hydrolase B, serine esterase	3807109	3.67
Liver annexin-like protein	7108713	-2.57
Mitochondrial processing protease	397699	-2.57
Mitochondrial trifunctional protein, alpha subunit precursor	148747393	-2.47
N-acetylneuraminate pyruvate lyase 2	157822207	-2.17
Precursor polypeptide (AA -18 to 547)	56899	3.67
Preproapolipoprotein A-I	55747	-2.37
Protein disulfide-isomerase A3 precursor	8393322	-2.57
Protein kinase inhibitor p58 precursor	11560030	1.57
Pyruvate carboxylase precursor	31543464	-1.57
Rieske Fe-S protein precursor	206681	-8.71
Serine protease inhibitor A3N	2507388	-3.07
Stress-70 protein, mitochondrial	116242506	-2.17
Sulfite oxidase	294639	1.57
T-kininogen 1	60392582	-4.87

\* Accession no. is from NCBI protein database (<ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz>)