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Altered mitochondrial function and genome frequency post exposure to γ -radiation and bystander factors

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ALTERED MITOCHONDRIAL FUNCTION AND GENOME FREQUENCY POST
EXPOSURE TO γ RADIATION AND BYSTANDER FACTORS

Running Title: Mitochondrial function post irradiation.

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Abstract

Purpose: To further evaluate irregular mitochondrial function and mitochondrial genome damage induced by direct γ irradiation and bystander factors in human keratinocyte (HPV-G) epithelial cells and hamster ovarian fibroblast (CHO-K1) cells. This is as a follow up to our recent reports of γ -irradiation induced loss of mitochondrial function and mitochondrial DNA (mtDNA) damage.

Materials and Methods: Mitochondrial function was evaluated post direct radiation and irradiated cell conditioned medium (ICCM) by determining: activity of the individual complexes of oxidative phosphorylation (OxPhos); mtDNA-encoded protein synthesis; mitochondrial genome frequency and mtDNA damage.

Results: Mitochondria show a loss of OxPhos enzyme function as early as 4 hours post treatment with recovery observed 12-96 hours in some but not all complexes demonstrating a non-uniform sensitivity to γ radiation. We also identified irregular mtDNA-directed protein synthesis. Long range Polymerase Chain Reaction (PCR) analysis identified mitochondrial genome damage and real time PCR identified increases in mitochondrial genome frequency.

Conclusions: The study reaffirms the sensitive nature of mitochondria to both low-level direct radiation exposure and radiation-induced bystander factor mediated damage. Furthermore, we report for the first time, the loss of function in the enzymes of OxPhos post exposure to bystander factors and identify altered mtDNA-directed protein synthesis post both direct radiation and bystander factors.

Introduction

The bystander effect may be observed when cells, not exposed to radiation, display radiation-like damage, when in the vicinity of irradiated cells or exposed to medium from irradiated cells. Previous studies have reported these bystander factors to induce chromosome aberrations (Lorimore *et al.* 2008), micronuclei induction (Prise *et al.* 1998), changes in gene expression (Azzam *et al.* 1998), sister chromatid exchanges (Nagasawa and Little 1992), apoptosis and reproductive death (Lyng *et al.* 2000), increases in ROS (Narayanan *et al.* 1999) and genomic instability in future generations of cells (Seymour and Mothersill 1997, Wright 1998). At present the bystander factor has yet to be characterised, though it is considered to be a cellular stress response or damage signal resulting from a range of signal transduction pathways (Lyng *et al.* 2002, Lyng *et al.* 2006, Zhou *et al.* 2005, Zhou *et al.* 2008). Furthermore it is now evident that the mitochondria play a significant role in both mediating and regulating the bystander effect (Tartier *et al.* 2007, Chen *et al.* 2008, Zhou *et al.* 2008). Evidence has accumulated that at very low doses of radiation, any subsequent damage is predominantly as a result of the release of bystander factors and the direct effects at such low doses are negligible (Seymour and Mothersill 2000, Mothersill *et al.* 2004). There are three main approaches used to demonstrate bystander effects: (i) cells may be exposed to low fluences α -particles such that only a small fraction of cells are traversed by a radiation track (Nagasawa and Little 1992); (ii) a microbeam may be used to traverse a single cell or a specific region of a cell (Prise *et al.* 1998); (iii) Irradiated cell conditioned medium (ICCM) is transferred to unirradiated cells (Mothersill and Seymour 1997). The latter method employing ICCM is employed in this study.

The mitochondrial genome is the only other source of genetic material outside the nucleus and is a maternally inherited closed circular double stranded structure (Taanman, 1999). Although the genome encompasses a minute fraction of the total genetic material in a cell (0.5-1%), any damage or alteration to it can still have serious implications for a cell's viability and / or survival. Mitochondrial DNA (mtDNA) is made up of almost entirely of coding regions, it lacks a protective histone coat, undergoes limited proof reading and repair when copied and is in close proximity to a rich source of reactive oxygen species (ROS) via Oxidative Phosphorylation (OxPhos). All of these factors contribute to the 10-20 fold greater mutation rate observed in this genome compared to the nuclear genome (Gray 1999). Ninety percent of cellular oxygen is consumed in OxPhos (Karthikeyan and Resnick 2005), where electrons are passed from complex to complex and like any other biological system, this is never 100% efficient and electrons are periodically lost to the matrix. These electrons that leak may interact with molecular oxygen to form superoxide radicals ($O_2^{\cdot -}$) which are converted into ROS. ROS has been linked to an increase in mitochondrial mass (Limoli *et al.* 2003, Lee *et al.* 2005, Spodnik *et al.* 2002). Malakhova *et al* (2005) found a significant increase in mtDNA copy number in post mitotic brain and mitotically active spleen tissue of mice 24-72 hours post 3 Gy γ irradiation. ROS have also have been shown to have a role in perpetuating the bystander effect (Lyng *et al.* 2002, Iyer and Lehnert 2000). Wang *et al* (2007) reported a 2-3 fold increase in mitochondrial mass and 4-5 fold increase in mtDNA in the progeny of low-dose irradiated HepG2 cells. Kim *et al* (2006) showed that mitochondrial dysfunction had a role in maintaining oxidative stress. These authors demonstrated that this was not due to an increase in mitochondrial numbers or increases in mtDNA levels. Instead they found significant decreased levels of state 3 respiration, manganese superoxide

dismutase (MnSOD) activity, cytochrome c oxidase (Complex IV) activity and increases in H₂O₂ levels were observed. These authors concluded that alterations to the Electron Transport Chain (ETC), such as mutations or altered gene expression could inhibit oxygen uptake and an increase in ROS could inactivate MnSOD, all of which are contributing factors in oxidative stress.

The mitochondrial genome is controlled by approximately 1500 genes in total with only 37 of these encoded by mtDNA. These include 22 transfer RNAs (tRNAs), 2 ribosomal RNAs (rRNAs) and 13 enzyme subunits that make up part of the OXPHOS chain. Complexes I, III, IV and V contain both nuclear DNA (nDNA) and mtDNA encoded sub-units, while only complex II is entirely encoded by nDNA and therefore mtDNA damage will manifest in this pathway. Indeed, alterations in the respiratory system and mtDNA appear to be a general feature in malignant cells (Carew and Huang 2002). Damage leads to OxPhos dysfunction, which gives rise to defects in mitochondrial ATP production and numerous disorders with the main tissues affected being those of higher energy demand such as skeletal muscle and the nervous system. Simonnet *et al* (2003) reported that complex I activity and protein content was significantly reduced in contrast to other enzyme complexes in renal oncocytomas benign tumours and suggested that deficiency in complex I could be the contributor for the initiation of mitochondrial biogenesis. Complex I dysfunction is one of the main causes of mitochondrial disease and its deficiency can result in disorders such as Parkinson's disease and Leigh's syndrome (Almeida and Medina 1998, Visch *et al.* 2004, Palacino *et al.* 2004). Simonnet *et al* (2002) reported up to a 5 fold increase in the mitochondrial mass marker citrate synthase activity correlating with tumour aggressiveness in patients suffering from renal cancer. These results were in parallel with a 7 fold increase in complex IV activity,

while II, III and V were only slightly increased, suggesting that mitochondria increase to compensate for an overall decrease in ATP reduction through OxPhos. Although Savanger *et al* (2001) showed that oxygen consumption rates were defective despite increased mitochondrial accumulation in oncocytoma thyroid oxphilic tumours. Rossignol *et al* (2003) reported that glucocorticoid-treated mice suffering from a mitochondrial myopathy had an increase in mitochondrial mass and these authors suggested that this was a mechanism to compensate for an oxidative defect, facilitating maximum oxygen uptake and a higher yield of ATP within the cell.

In the present study, HPV-G and CHO-K1 cells were examined to further determine the impact of direct irradiation and bystander factors on mitochondrial function, specifically the activity of OxPhos enzyme complexes, mtDNA-encoded polypeptide synthesis and mtDNA integrity. This study is a continuation of previous work by this group on mitochondrial damage induced by both direct irradiation and ICCM that included mtDNA mutations and a novel mtDNA deletion (Murphy *et al.* 2005) and altered mitochondrial mass and mitochondrial oxygen consumption (Nugent *et al.* 2007).

Materials and Methods

Cell Culture

Two cell lines were employed in this study: a human keratinocyte epithelial cell line (HPV-G) derived from human neonatal foreskin transfected with the HPV 16 virus (Pirisi *et al.* 1998), supplied as a kind gift from Dr. J. Di Paolo (NIH, Bethesda, MD) and a spontaneously transformed Chinese Hamster ovarian cell line (CHO-K1), (European Collection of Cell

Cultures, Salisbury, UK) (Kao and Puck 1968). HPV-G cells were maintained in Dulbecco's Modified Eagle Nutrient Mixture (DMEM)/ F12 (Sigma, Dorset, UK), supplemented with 10 % foetal calf serum (FCS) (Gibco, Biocult Irvine, Scotland), 20 mM L-Glutamine (Gibco), 1U/ml penicillin/ streptomycin (Gibco) and 1 µg/ ml hydrocortisone (Sigma). CHO-K1 cell line were maintained in Ham's Nutrient Mixture, F12 (Ham) (Sigma), supplemented with 12 % foetal calf serum (Gibco), 1 U/ml penicillin/ streptomycin (Gibco), 20 mM L-glutamine (Gibco) and 25 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (Gibco). Both cell lines were maintained in an incubator at 37 °C, 5 % CO₂ and 95 % humidity.

Direct Irradiation

Cells were grown to 70-80 % confluency in cell culture flasks. Cells were either sham irradiated or directly irradiated with doses of 5 mGy, 0.5 Gy or 5 Gy γ -radiation at room temperature using a Cobalt 60 teletherapy unit (St. Luke's Hospital, Rathgar, Dublin). The dose rate was either 1.8 Gy/ min at a source to flask distance of 80 cm (for 0.5 and 5 Gy) or 0.4 Gy/ min at a source to flask distance of 170 cm (for 5 mGy). The flasks were returned to the incubator immediately after irradiation and maintained in normal cell-culture conditions for analysis 4-96 hours later.

Exposure to Bystander Factors

Donor T-25 flasks (NUNC, Roskilde, Denmark) containing 5×10^5 cells per 5 ml medium were irradiated or sham irradiated at room temperature using a Cobalt 60 teletherapy unit (St. Luke's Hospital, Rathgar, Dublin) 1 hour post addition of fresh medium to each flask. The dose rate was either 1.8 Gy/ min at a source to flask distance of 80cm (for 0.5 and 5Gy) or 0.4Gy/ min at a source to flask distance of 170 cm (for 5 mGy). Flasks were exposed to either 0 Gy (sham), 5 mGy, 0.5 Gy or 5 Gy. Medium was removed from each flask 1 hour post irradiation and passed through a 0.22 μm sterile filter (Anachem, Luton, UK) to eliminate cells or debris from the ICCM. ICCM was transferred to un-irradiated cells grown to 70-80 % confluency in T-75 flasks (NUNC) for enzyme kinetic assays and mtDNA molecular analysis or in six wells plates for mitochondrial protein synthesis analysis. These ICCM recipient flasks were returned to the incubator and maintained in normal cell-culture conditions for analysis 4-96 hours later.

DNA isolation, quantification, separation and visualization

Total DNA was isolated using the Total DNA GenElute kit (Sigma) according manufacturer's instructions. DNA purity and concentration was confirmed by spectrophotometric analysis at 260 and 280 nm. Polymerase Chain Reaction (PCR) products were electrophoresed on a 0.8-1.2 % agarose gel supplemented with Ethidium Bromide. Bands were visualised, photographed, and band densities were calculated using a GeneGenius DNA imager and associated software (Syngene, Cambridge, UK).

Long Range PCR Amplification

20 µl PCR reactions were prepared containing 1x Readymix Extensor PCR Mastermix (ABgene, Epsom, UK) and 0.1 µM of GGCACCCCTCTGACATCC (forward) and TAGGTTTGAGGGGGAATGCT (reverse) primers along with 5 ng template DNA. Reactions were put through an initial denaturing step, of 94 °C for 30 seconds followed by 15 cycles of 94 °C for 10 seconds; 56 °C for 2 minutes and 68 °C for 8 minutes. A further 20 cycles at 94 °C for 10 seconds; 56 °C for 2 minutes and 68 °C for 8 minutes (+10 seconds per cycle), and a final elongation step of 68 °C for 15 minutes. PCR cycles were terminated at 25, 20, 18, and 15 cycles to ensure amplification was terminated in the linear range. Initially elongation times were systematically reduced in an attempt to bias PCR amplification of mutant genomes (containing deletions) in preference over wild-type genomes, however no additional polymorphic bands were observable.

Real Time PCR

Relative mitochondrial genome frequency was determined by Real time PCR using a Lightcycler 1.5 (Roche, West Sussex, UK). A 152 bp conserved region of the mitochondrial genome was amplified using ATCAACTGGCTTCAATCTACTTCTC (forward) and GTAAATCTAAAGACAGGGGTTAGGC (reverse) primers. This was normalized against the amplification of a 155 bp region of the nuclear encoded β actin gene using CGGCAGAAGAGAGAACCAGTG (forward) and TGACTGGCCCGCTACCTCTT (reverse) primers.

PCR reaction volumes of 15 μ l were prepared containing 1x Brilliant[®]SYBR[®] Green QPCR master mix (Stratagene, Leicester, UK), 0.5 μ M of forward and reverse primer and 5 ng template DNA. Reactions were put through an initial denaturing step at 95 °C for 10 minutes; followed by 40 cycles of 95 °C for 30 seconds, 55 °C for 1 minute and 72 °C for 30 seconds. This was followed by standard melt curve analysis. A standard curve was performed to determine primer efficiency using serial 1 in 10 dilutions of template. Cycle threshold (Ct) values were recorded and converted to relative quantification ($E^{-\Delta\Delta C_t}$, where E = efficiency and Ct = cycle threshold) according to Pfaffl (2001).

Enzyme Kinetics Assays

Cells were harvested and resuspended in Phosphate Buffered Saline (PBS) solution supplemented with 10 % glycerol, and stored at -20 °C. Protein concentration was determined using a modification of the Bradford assay (Bradford 1976). Assays were performed using minor modifications of the OxPhos enzyme kinetics assays described by James *et al* (1996).

Complex II-III analysis

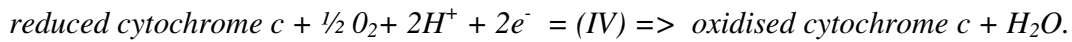
The measurement of complex II-III is based on the reaction:



Reactions for Complex II-III were prepared with 50 mM KH_2PO_4 , 0.1 mM Ethylenediaminetetraacetic acid (EDTA), pH7.4, 25 mM Succinate, 2 mM KCN, 0.4 mM Rotenone and 0.1 mM oxidised cytochrome c equilibrated to 30 °C for 5 minutes prior to the addition of 10 μ l sample (20-200 μ g) total cell protein. Absorbance was recorded at 550 nm ($\epsilon_{550} = 21.1 \text{ nM}^{-1}\text{cm}^{-1}$). Complex II-III activity was confirmed using 100 μ M myxothiazol.

Complex IV Analysis

The measurement of complex IV is based on the reaction;

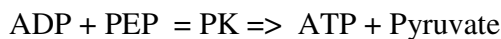
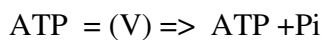


Reactions for complex IV contained 200 mM Tris, 10 μ M EDTA, pH 7.5, 0.4 mM Rotenone, 0.6 μ M Antimycin A₁ and 0.1 mM reduced cytochrome c and equilibrated to 30 °C for 5 minutes prior to the addition of 10 μ l sample (20-200 μ g) total cell protein. Absorbance was recorded at 520 nm ($\epsilon_{520} = 27.7 \text{ nM}^{-1}\text{cm}^{-1}$). Complex IV activity was confirmed using 2 mM KCN .

Complex V Analysis

The measurement of complex V is based on three reactions;

The first reaction involves ATP is hydrolysed to ADP in the presence of complex V and this ADP is reformed by a reaction with phosphoenolpyruvate (PEP) in the presence of pyruvate kinase (PK) to form ATP and pyruvate. The second reaction involves pyruvate being converted to lactate by lactate dehydrogenase (LDH) and the oxidation of NADH to NAD is measured at 340 nm ($\epsilon_{340} = 6.811 \text{ nM}^{-1}\text{cm}^{-1}$).



Reactions for complex V contained 100 mM Tris, 50 mM KCl, 2 mM MgCl₂, 0.2 mM EDTA, pH 8, 0.125 mM NADH, 2.5 mM MgATP, 4 U PK, 10 U LDH, 2 mM KCN, 0.4 mM

Rotenone, 5 μM Antimycin A₁ and 1 mM PEP and were equilibrated to 30 °C for 5 minutes prior to the addition of 10 μl sample 20-200 μg total cell protein. Absorbance was recorded at 340 nm ($\epsilon_{340} = 6.811 \text{ nM}^{-1}\text{cm}^{-1}$) until no fluctuation was seen (MgATP contains a small amount of ADP; therefore any fluctuation observed is ADP conversion to ATP). Complex V activity was confirmed using 10 μM oligomycin.

MtDNA-Encoded Protein Synthesis Analysis

The mitochondrial protein synthesis activity was measured following techniques described by King *et al* (1992) with some modifications. Cells were maintained at 37 °C for 2 hours in a suspension of 50 mM succinate, 20 mM ADP, 133 μM amino acid mix (minus lysine), 1 mg/ml cycloheximide and 1 μl biotinylated tRNA^{lys} (Promega, Southampton, UK) in PBS buffer.

Biotin incorporation into nascent mtDNA-encoded proteins was stopped by the addition of 10 mM Lysine along with a further incubation at 37 °C for 5 minutes to prevent partial synthesis of biotinylated nascent proteins. Cells were resuspended in PBS buffer and Laemlii Buffer was added to each sample (final concentrations 2 %, Sodium Dodecyl Sulphate (SDS), 10 % glycerol, 5 % 2-mercaptoethanol, 600 mM Tris pH 6.8 and bromophenol blue). Protein concentration in each sample was determined using a modification of the Bradford assay (Bradford 1976) and samples diluted as required to ensure equal loading. Samples were heated to 100 °C for 10 minutes then 20 μl of each loaded on an SDS polyacrylamide gel containing a 15 % separating layer and a 5 % stacking layer with running buffer (0.25 M Tris, 1.92 M Glycine and 1% SDS, pH 8.3) at ~6 mA until sufficient separation was achieved. Proteins were transferred, by semi-dry western blot, onto a Polyvinylidene Fluoride (PVDF)

membrane (AGB Scientific, Dublin, Ireland) using Transfer Buffer (0.39 mM Glycine, 48 mM Tris, 0.037% SDS and 20% methanol). The membrane was blocked using Tris Buffered Saline with Tween (TBST) buffer (100 mM Tris, 150 mM NaCl and 0.1 % Tween20, pH 7.5) over night. The membrane was then washed using a 1:10,000 dilution of streptavidin-HRP conjugate/ TBST for 1 hour to allow binding to occur. Biotinylated proteins were visualised using chemiluminescence and autoradiography. Changes in protein synthesis were qualitatively identified. Protein identity was predicted using their known molecular weights (kDa). MtDNA-encoded origin of bands shown was confirmed using 100 μ M chloramphenicol supplement in the cell suspension (data not shown).

Statistics

Values are expressed as the mean \pm standard error of the mean (SEM). Data are representative of three or more experiments. The multiple measures analysis of variance (ANOVA) was performed to determine significance, and values were considered significant if $p \leq 0.05$.

Results

Enzyme Kinetics Analysis

Analysis of CHO-K1 cells showed that enzyme activity was affected from 4 hours post 0.5 and 5 Gy direct exposure and ICCM (figure 1). Complex II-III activity post 5 Gy direct radiation and ICCM recovered to control levels by 12 hours post exposure. Recovery of complex V activity to control levels was observed 96 hours post direct exposure and ICCM exposure. Complex IV activity was observed as less than control 4 and 12 hours post ICCM

and recovered to levels similar to control 24 hours post ICCM and above control by 96 hours post ICCM. Complex IV activity post direct exposure was significantly less than control at all time points studied (figure 1).

Complex II-III activity in exposed HPV-G cells was similar to control 4 hours post 5 Gy direct irradiation and activity post 5 Gy ICCM remained similar to control through 12 hours but increased significantly above control 24 hours post exposure and this increase was sustained 96 hours post exposure. Activity post 5Gy direct exposure showed a significant though transient increase through 12 and 24 hours post exposure, however activity fell significantly below control 96 hours post exposure (figure 2A).

Complex IV activity in exposed HPV-G cells was significantly lower than control 4 hours post 5 Gy direct irradiation, a reduction that was sustained through 12, 24 and 96 hours post direct exposure. Activity post 5 Gy ICCM exposure showed a significant though transient reduction compared to control 4 hours post exposure, though recovered by 12 hours, a recovery that was sustained through 24 and 96 hours post exposure (figure 2B).

Complex V activity in exposed HPV-G cells was similar to control 4 to 24 hours post 5 Gy ICCM but was significantly less than control 96 hours post exposure. Activity post 5 Gy direct exposure showed a significant though transient reduction compared to control 4 hours post exposure, though recovered by 12 hours, a recovery that was sustained through 24 and 96 hours post exposure (figure 2C). Indeed the effects of 5 mGy and 0.5 Gy irradiations were similar to that of 5 Gy when measured 4 hours post exposure. A summary of direct radiation and bystander effects on the enzymes of OxPhos of both cell lines is provided in table 1.

Mitochondrial In organello Protein Synthesis Analysis

CHO-K1 cells post direct treatment (figure 3A) show a decrease in mitochondrial protein synthesis observed as early as 4 hours post 5Gy direct exposure, which increased through 12 to a level above control 24 hours later. However, there was a decrease in synthesis compared to that of control 96 hours post 5 Gy treatment. Reduced synthesis was observed with decreasing dose at 96 hours post exposure, with a reduction in synthesis post 5 mGy such that the majority of polypeptides were either not synthesised at all or synthesised at a level too low to be detected (figure 3A).

ICCM treatment of CHO-K1 cells had less pronounced effects on mitochondrial protein synthesis, though effects were observable as early as 4 hours post treatment where ND1 and ND6 synthesis was reduced. This reduction appeared transient as synthesis recovered through 12, 24 and up to 96 hours post exposure (figure 3B).

HPV-G cells that were exposed to direct irradiation showed no change in mitochondrial protein synthesis at any time point post 5 Gy (figure 4a). Interestingly, at 96 hours post 0.5 Gy altered synthesis was observed, with CO1 protein appearing greatly reduced. Furthermore, at 96 hours post 0.5 Gy irradiation there were two irregular sized proteins synthesised (of approximately 50 and 15 kDa) and these proteins were not observed in any of the other treated samples or in the control (figure 4A).

ICCM treated HPV-G cells showed no significant changes in protein synthesis until 96 hours post exposure. At this time, cells post 5Gy ICCM showed a marked increase in protein synthesis, whereas cells post 0.5 Gy and 5mGy ICCM showed reduced protein synthesis (figure 4B).

Long Range PCR Analysis

HPV-G cells were exposed to direct irradiation and ICCM as described previously. Long range PCR was carried out to identify non-specific large deletions. An equal amount of DNA was added to each PCR reaction, using a primer set to amplify almost the entire genome. No large deletions were found, although reduced PCR-product band intensities were an observable feature 96 hours later, however only post higher doses of direct radiation. This reduced PCR efficiency of only long range PCR product, is suggested to result from global non-specific mtDNA damage (as no similar loss was observed in short product amplification as shown subsequently in figure 6). No loss of PCR efficiency was observed at 12 or 24 hours post treatments (figure 5A) suggesting a delay in this damage being expressed. At 96 hours post direct treatment, efficiency of PCR product amplification was seen to be severely reduced post 0.5 Gy and 5 Gy (figure 5A). Interestingly, there is no loss of PCR efficiency post 5 mGy direct exposure (figure 5A). In ICCM treated samples it was observed that at no time or dose, was any damage load to mtDNA sufficient to impair PCR (figure 5).

Real Time PCR Analysis

HPV-G cells were exposed to direct irradiation and ICCM as described previously. Real time PCR was carried out to determine any changes in mitochondrial genome frequency. A significant increase in mitochondrial genome frequency was first observed in HPV-G cells 24 hours post direct and ICCM treatment. However, ICCM values were lower than those of direct irradiation (figure 6A). At 96 hours post direct treatment the increase in genome frequency was maintained, although this was not comparable with results at 96 hours post ICCM

treatment, where it was observed that mitochondrial genome frequency was not significantly greater than control. This would infer that the increase in mitochondrial genome number observed post ICCM is transient where as the increase post direct radiation is sustained (figure 6A).

In HPV-G cells 96 hours post direct exposure to 0.5 Gy and 5 Gy, mitochondrial genome frequency was significantly greater than that of control cells. However, the most noticeable effect was observed following 5 mGy exposure, where mitochondrial genome frequency was approximately 3.5 fold that of control cells (figure 6B). An increase in mitochondrial genome frequency was also observed 96 hours post exposure to ICCM treated cells, though not as pronounced as in HPV-G cells 96 hours post 5 mGy direct irradiation (figure 6B).

Discussion

OxPhos enzyme complex assays are a sensitive tool for identifying changes in mitochondrial function. Previous studies have employed this to examine mitochondrial function in mitochondrial myopathy and age related studies (Palacino *et al.* 2004, Chinnery *et al.* 2002, Schon *et al.* 1997, Lesnefsky and Hoppel 2003, Blanco *et al.* 2004). Complex I analysis was excluded as this assay cannot be accurately performed in whole cell fractions due to non-mitochondrial NADH-quinone oxidoreductase activity and limited permeability of substrates to complex I. The complex I assay can only be accurately performed on mitochondrial fractions (Chretien *et al.* 2003). Mitochondrial fraction preparation is very inefficient from cultured cells and would have necessitated the use of over 10 times the amount of cells. Complex II and III assays were pooled for analysis as the complex III activator, ubiquinone, is prohibitively expensive and problematic to source. Instead, by pooling complex II and III

together, it is possible to activate complex II to drive the ubiquinone source that is already in the mitochondrial membrane for complex III analysis. In this study an interesting pattern was identified in the sensitivity of the individual enzymes of OxPhos to radiation. Complex IV activity was very low post direct exposure and did not recover with time. This result compares favourably with previous studies on age associated mitochondrial dysfunction (Chinnery *et al.* 2002, Schon *et al.* 1997, Lesnefsky and Hoppel 2003). Schon *et al.* (1997) reported that all complexes were affected by age and was most pronounced in complex IV. This study indicates that the sensitivity of complex IV to damage by γ radiation is much more pronounced than the other enzyme complexes. Letellier *et al.* (1994) reported that complex IV activity had to exceed a critical value of 75% inhibition before a decrease in mitochondrial respiration could be observed. Muscle fibre studies have confirmed that the proportion of mutated mtDNA varies between muscle fibres and only mtDNA containing mutations over threshold levels displayed a deficiency in complex IV activity (Petruzzella *et al.* 1994, Moslemi *et al.* 1998). In CHO-K1 cells, activity decreased as a dose dependent response at 4 hours post treatment in all enzyme complexes which was followed by a recovery in enzyme function occurring at 12 hours post treatment with the exception of complex IV post direct treatment. In HPV-G cells, only complex V analysis at 4 hours post direct treatment showed a similar dose dependent response pattern as seen in CHO-K1 cells. HPV-G analysis for complex II-III showed that enzyme activity levels had an almost immediate increase at the lower doses in both direct and ICCM treated cells with delayed transient increase observed 12 and 24 hours post 5Gy. Decreases in OxPhos activity along with increases in mitochondrial mass have been reported. Blanco *et al.* (2004) showed a correlation between ageing individuals over 40 years suffering from osteoarthritis and mitochondrial dysfunction. These

authors reported that significant decreases were observed in complexes I, II and III activity along with increased mitochondrial mass, apoptotic bodies, B-cell leukaemia/lymphoma 2 protein (bcl-2) expression, Caspase 3 expression and nitric oxide. A decrease in the mitochondrial membrane potential was also noted. It is unclear from the present however if the observed loss of mitochondrial OxPhos enzyme function, both transient and apparently more long term, in the case of Complex IV in directly exposed cells, may be attributable to radiation injury or indeed may represent a mitochondrially-mediated adaptive response to radiation injury as more recently mitochondrial dysfunction has been demonstrated to induce G1-S arrest (Owusu-Ansah *et al.* 2008). Indeed mitochondrial function is now understood to play a much more significant role in cell cycle regulation than has been understood heretofore (Finkel and Hwang 2009).

Mutations of mtDNA have been established as a primary contributor to mitochondrial disease (Jones *et al.* 2008). Wardel *et al.* (2003) observed malignant cells in colorectal cancer had an altered phenotype that was due to mutations in the mtDNA.

Mutations may impair synthesis or a change in translation of one, several, or all of the thirteen polypeptides that make up OxPhos (DiMauro 2004, Rossignol *et al.* 2003). Mutations in mtDNA may result in the production of a premature stop codon, a random mutation at the ribosomal active recognition site, on a tRNA gene, rRNA gene or gene region coding for either the active or folding region of the protein. It has been shown that a single point mutation in human mitochondrial tRNA^{Met} induced the mitochondrial myopathy Leber's Hereditary Optic Neuropathy disease (Jones *et al.* 2008), resulting in severely abnormal mitochondria with OxPhos defects. In this study mitochondrial protein synthesis was used to assess irregular mitochondrial function. This technique has been used to identify

mitochondrial disorders such as Myoclonic Epilepsy with Ragged Red Fibres (MERRF) syndrome (Hanna *et al.* 1995) and Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like episodes (MELAS) syndrome (Chomyn *et al.* 1992). Yan *et al.* (2005) reported that the A1491G/C1409T mutation associated with deafness displayed reduced expression of Cytochrome b (Cytb) and Cytochrome c oxidase subunit I (CO1) and mitochondrial protein synthesis was almost completely abolished. In the present study, irregular mtDNA-directed protein synthesis was identified in both CHO-K1 and HPV-G cells post direct irradiation and ICCM treatment. The most noticeable observation using this technique was irregular synthesis as early as 4 hours post treatment in CHO-K1 cells and 96 hours post treatments in HPV-G cells. This suggested HPV-G cell response was not as immediate as observed in CHO-K1 cells. However, HPV-G cells appear more sensitive than CHO-K1 cells to lower doses, as proteins synthesis was severely reduced. Furthermore, in HPV-G cells 96 hours post 0.5Gy irradiation, CO1 and Cytochrome c oxidase subunit 2 (CO2) protein synthesis was affected. However, synthesis of two irregular sized proteins (~50 and 15kDa) was observed. One possible explanation could be the occurrence of a deletion spanning regions encoding both CO1 and CO2 (though this should have been observed by long range PCR analysis) or mutation(s) giving rise to a premature stop codon occurring in the polypeptide sequence of CO1 and CO2. In CHO-K1 cells a transient loss of mitochondrial protein synthesis was observed at 4 hours post treatment. These results are in agreement with our previous polarographic analysis (Nugent *et al.* 2007) which shows a significant though transient loss of oxygen consumption rates observed as early as 4 hours, while delayed effects were observed in the HPV-G cells. In samples, where changes in mtDNA-encoded protein synthesis were similar across all peptides synthesised, one of several factors may be involved: (i) variation in

mitochondrial mass; (ii) variation in mtDNA copy number; (iii) variation in transcription / translation rate; (iv) altered cell cycle status.

Long range PCR, did not identify any induced deletions, neither in directly irradiated cells nor ICCM treated cells, the purpose for which the analytical technique was originally selected. It is likely that this technique required a deletion frequency higher than that induced in any exposed cells in this study. Unexpectedly, though this technique did provide a probable marker for what we termed 'non-specific global mitochondrial genome damage'. This was characterised through the observation of an apparent loss of PCR efficiency that did not correlate with mitochondrial genome frequency data. This suggests that damage continued to accumulate post exposure, to a sufficient degree to reduce PCR efficiency 96 hours post exposure, but only post higher doses of direct radiation. This would suggest that both a threshold level of exposure is required as well as a threshold of time post exposure before this damage is manifest at a detectable level. When these results are compared to short-range product amplification, where we found that mitochondrial genome frequency increased, it may be concluded that this loss of long range PCR efficiency could be an effective marker for 'global mtDNA damage' most likely as a result of strand breaks in the mitochondrial genome.

Alterations in mitochondrial genome copy number are frequently observed in many cancers (Tan *et al.* 2006, Kurtz *et al.* 2004, Tseng *et al.* 2006, Yu *et al.* 2007). Tan *et al.* (2006) reported that an increase of mtDNA copy number in oesophageal cancer was found in some tumours while a decrease was found in others. These authors also reported that no significant correlation was observed between copy number and mtDNA mutation load. Instead there appeared to be an inverse relationship in mtDNA content and tumour size. However, other studies found that a significant decrease in mtDNA copy number was associated with cancers

such as breast cancer (Tseng *et al.* 2006, Yu *et al.* 2007), prostate cancer and colon cancer (Lee *et al.* 2007). In this study relative mitochondrial genome frequency was confirmed using real time PCR. These values are largely in parallel with our previous mitochondrial mass data (Nugent *et al.* 2007). Increases in mitochondrial mass will serve to counteract loss of function and recover ATP synthesis capacity. An increase in mitochondrial genome frequency occurs in a dose dependent manner after direct irradiation although is independent of ICCM treatment and most likely represents an adaptive response by the mitochondrial population to radiation-induced stress. The increase in genome frequency was also seen to increase in an inverse dose dependent manner with an approximate 3.5 fold increase observed after 5mGy direct irradiation. The increased response observed here correlates with that reported previously by our group on mtDNA deletion analysis (Murphy *et al.* 2005) which showed that mtDNA⁴⁸⁸¹ deletion was more frequent in cells post low-dose direct γ -radiation dose when examined 96 hours later. Furthermore, we did not observe any correlation between genome frequency and OxPhos enzyme complex activity and, given that mtDNA encodes only a small number of the peptides making up these enzyme complexes, this is not unexpected.

More recently, mitochondrial function has been shown to have an important role in the production of a bystander response. Mothersill *et al* (2000, 2002) has shown the energetic status of mitochondria, to be a critical factor in the bystander effect and furthermore, Tartier *et al* (2007) reported that mitochondrial function was required for both radiation and bystander factors to induce tumour protein 53 (p53) binding protein foci. Zhou *et al* (2008) and Hei (2006) suggest a loss of mitochondrial molecular components make individual cells more susceptible to damage.

In general, the mitochondrial population of CHO-K1 cells were observed in this study to be more radiosensitive than those of HPV-G cells particularly in terms of time between exposure and damage being manifest. However HPV-G cells appeared more sensitive to the lower exposure doses. Furthermore, it has also to be considered that the bystander is more pronounced in irradiated HPV-G cells than in irradiated CHO-K1 cells (Mothersill *et al.* 2002, Vines *et al.* 2008). The difficulty in measuring bystander-induced mitochondrial changes most not be understated, given that that the energetic status of mitochondria, namely their capacity for ATP synthesis, has been previously observed as a potential critical factor in the bystander effect (Mothersill *et al.* 2000). Differences between the responses of the mitochondrial populations of CHO-K1 and HPV-G cells are therefore likely to be attributable to the specific cell-lines and not to their radiosensitivity or bystander factor sensitivity.

It must also be noted that there was a significant observable effect at every radiation dose employed in this study and even 5 mGy was not too low to induce a mitochondrial effect in both cell types. However data presented in the present study do not entirely concur with trends seen in previous analyses of low level radiation and bystander effects, namely that the strength of the bystander effects appear similar irrespective of the original exposure dose (Prise *et al.* 1998, Seymour and Mothersill 2000) and warrants future investigation.

When mitochondrial dysfunction and DNA damage observed in cells exposed to direct radiation are considered in the context of previous clonogenic analysis of these cell types exposed to 5Gy direct γ radiation, where cell death was equal in both HPV-G and CHO-K1 cells (Mothersill *et al.* 2000, Vines *et al.* 2008), no direct correlations may be drawn. This suggests that the radiosensitivity of cells as determined by clonogenic survival is not a good indicator of the radiosensitivity of the mitochondria contained therein. Determining the

radiosensitivity of these sensitive organelles would be better served by employing such endpoints as used in this study along with Polarography (Nugent *et al.*, 2007), mtDNA mutation analysis (Murphy *et al.*, 2005) and oxidative stress analysis. Indeed Fisher and Goswami (2008) observed that radioresistance in human pancreatic cancer cells was regulated by antioxidant enzyme activity targeted to mitochondria.

Another striking result that we report and to the best of our knowledge has not previously been reported before, is the development of a potential marker for what we termed 'non-specific global mitochondrial DNA damage' through identifying a decrease in PCR efficiency that cannot be attributed to reduced template. It is likely that the problematic correlation of observed mitochondrial dysfunction with mtDNA damage is mainly due to the complexity of this unique genome including the many point mutations and deletions incurred by it and its multi-heterogenic nature. Overall, results from this study suggest that there may be grave implications for the long term viability of mitochondria contained in the distant progeny of cells that survive radiation, given that the mitochondrial genome has the ability to carry significant heterogeneity without loss of function. Eventually though, a threshold will ultimately be reached beyond which the fate of these cells is uncertain and this includes the cell populations in this study that received very low doses comparable to that delivered by Computerized Axial Tomography (CAT) scans and other radiation-based routine medical diagnostics. There is also significant interest among many researchers in harnessing the bystander phenomenon for cancer therapeutic gain (reviewed by Prise *et al.* 2009) and which shows great potential, however the present study and recent studies by the authors (Murphy *et al.* 2005, Nugent *et al.* 2007) indicates that such studies must factor the sensitivity of

mitochondria to both low dose direct radiation and bystander factors and the potential of mtDNA to accumulate damage with time, long-term post exposure.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Figure 1

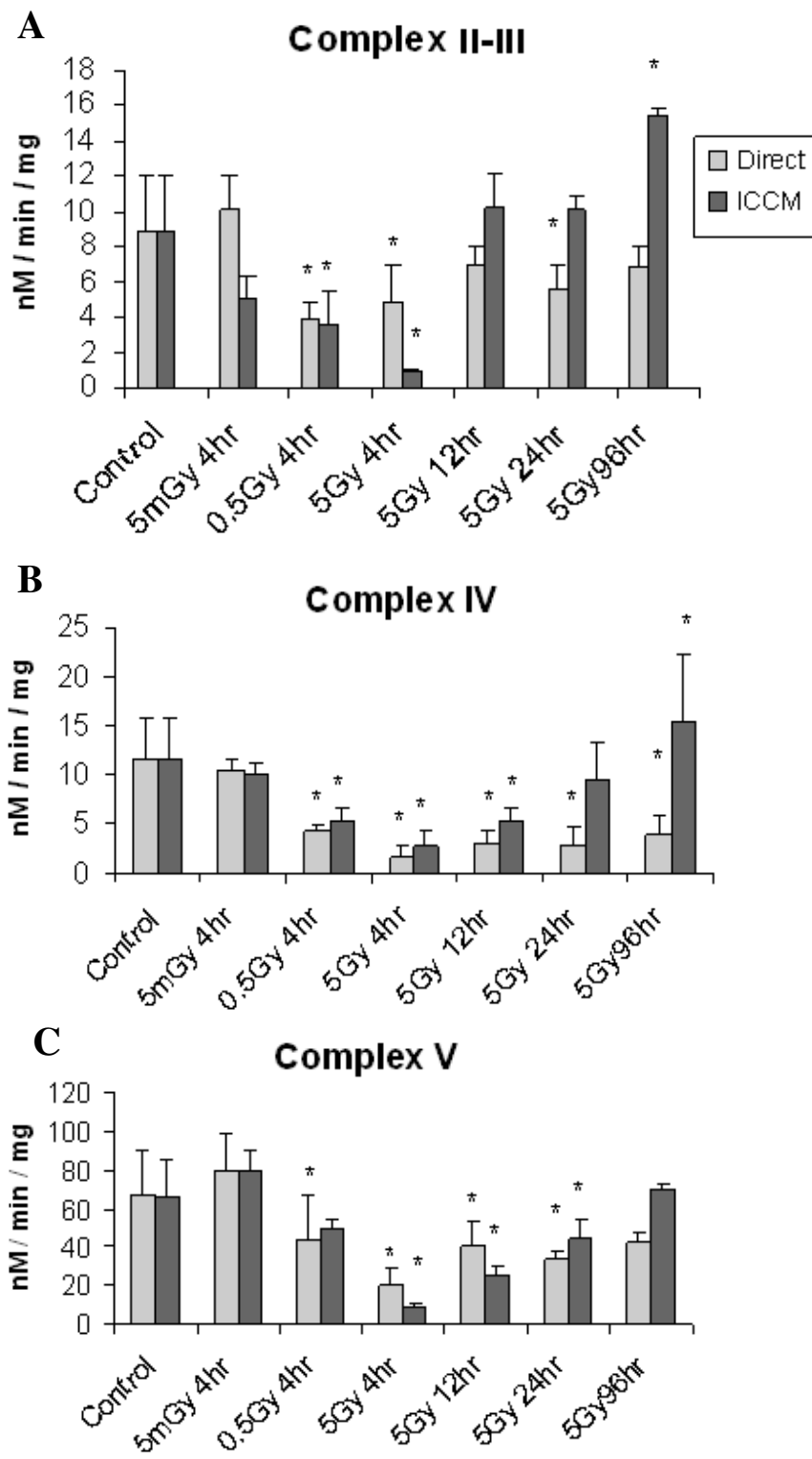


Figure 2

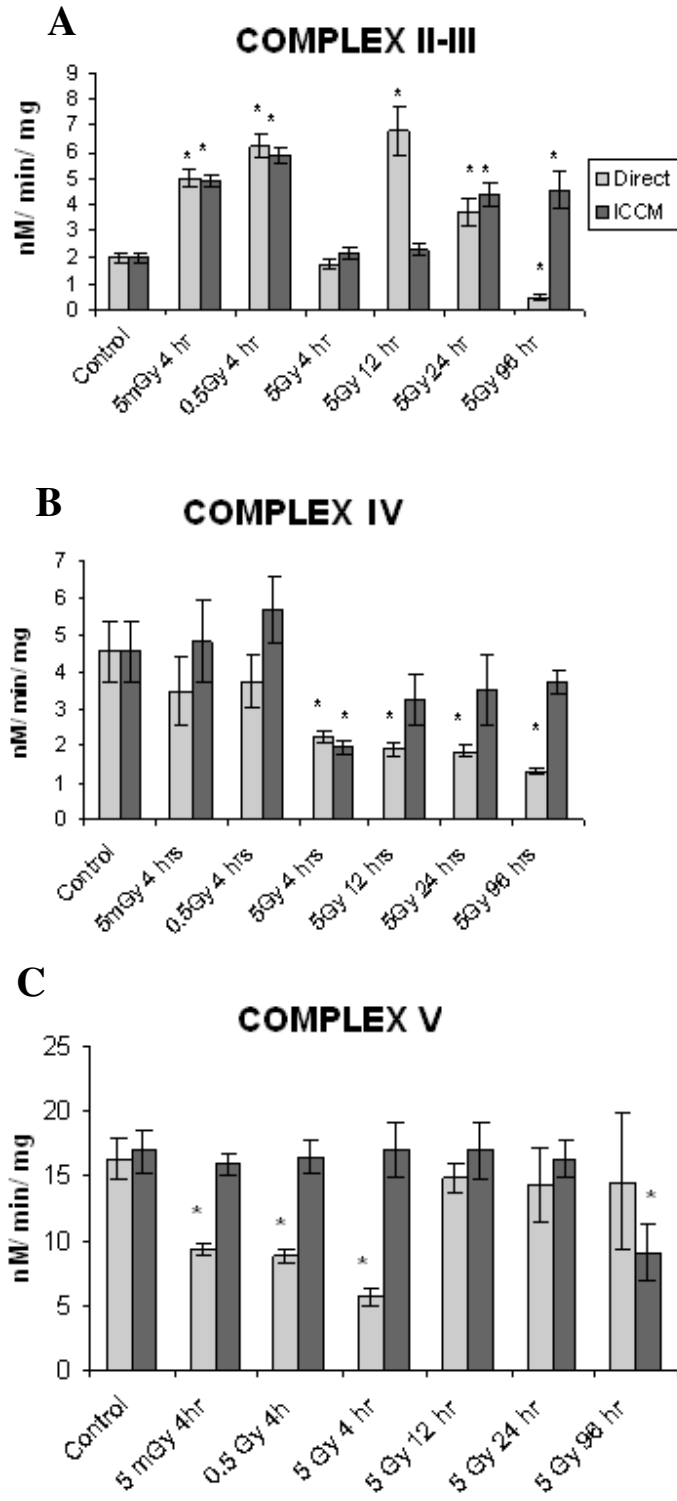


Table 1.

A	CHO-K1 cells 4 hours post			5Gy direct exposure			
	Direct	5mGy	0.5Gy	5Gy		4 hours	12 hours
II-III	↔	↓	↓		II-III	↓	↔
IV	↔	↓	↓		IV	↓	↓
V	↔	↓	↓		V	↓	↓

B	CHO-K1 cells 4 hours post			5Gy ICCM exposure			
	ICCM	5mGy	0.5Gy	5Gy		4 hours	12 hours
II-III	↔	↓	↓		II-III	↓	↔
IV	↔	↓	↓		IV	↓	↓
V	↔	↓	↓		V	↓	↓

C	HPV-G cells 4 hours post			5Gy direct exposure			
	Direct	5mGy	0.5Gy	5Gy		4 hours	12 hours
II-III	↑	↑	↔		II-III	↔	↑
IV	↔	↔	↓		IV	↓	↓
V	↔	↔	↓		V	↔	↔

D	HPV-G cells 4 hours post			5Gy ICCM exposure			
	ICCM	5mGy	0.5Gy	5Gy		4 hours	12 hours
II-III	↑	↑	↔		II-III	↔	↔
IV	↔	↔	↓		IV	↓	↔
V	↔	↔	↔		V	↔	↔

Figure 3

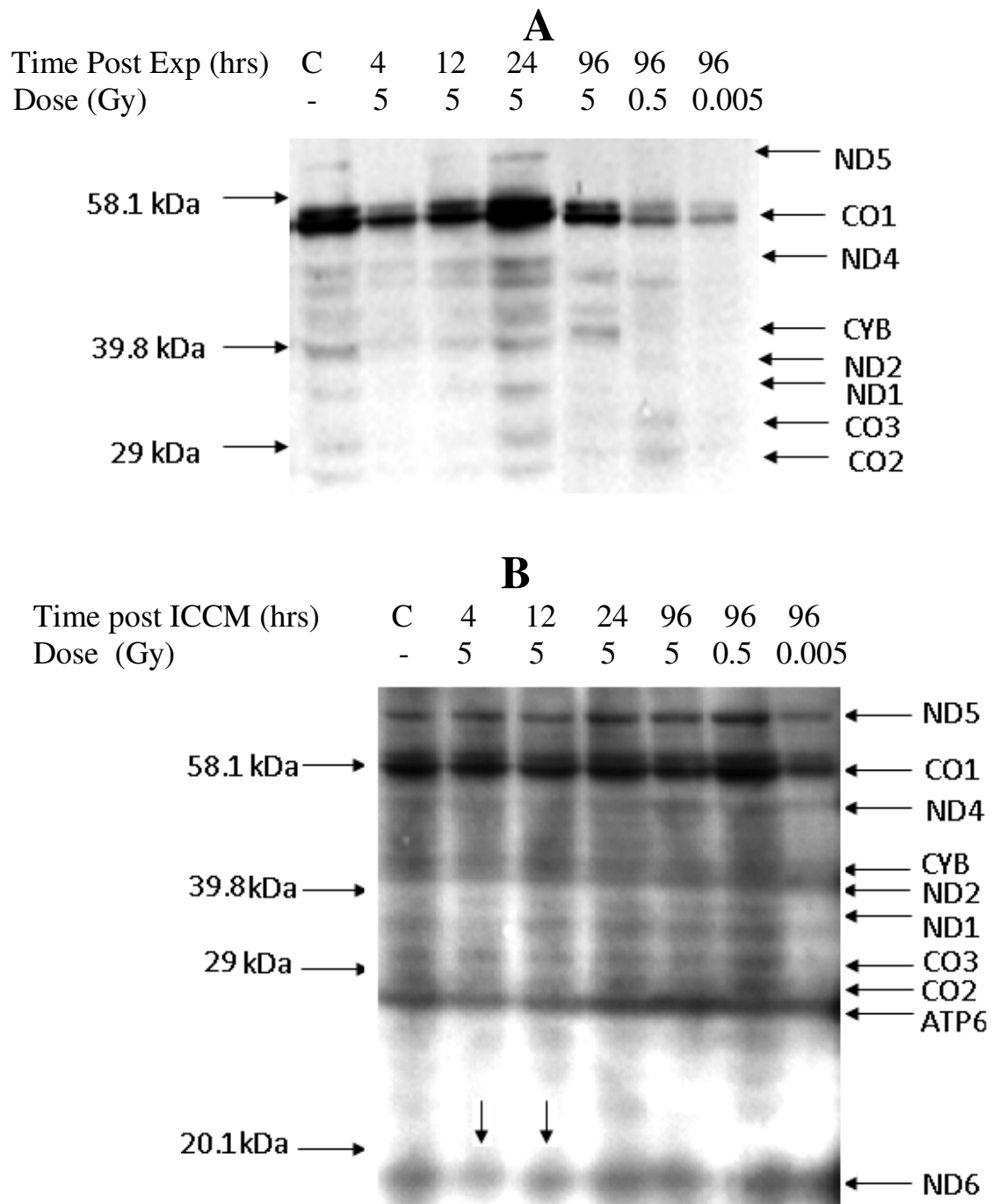


Figure 4

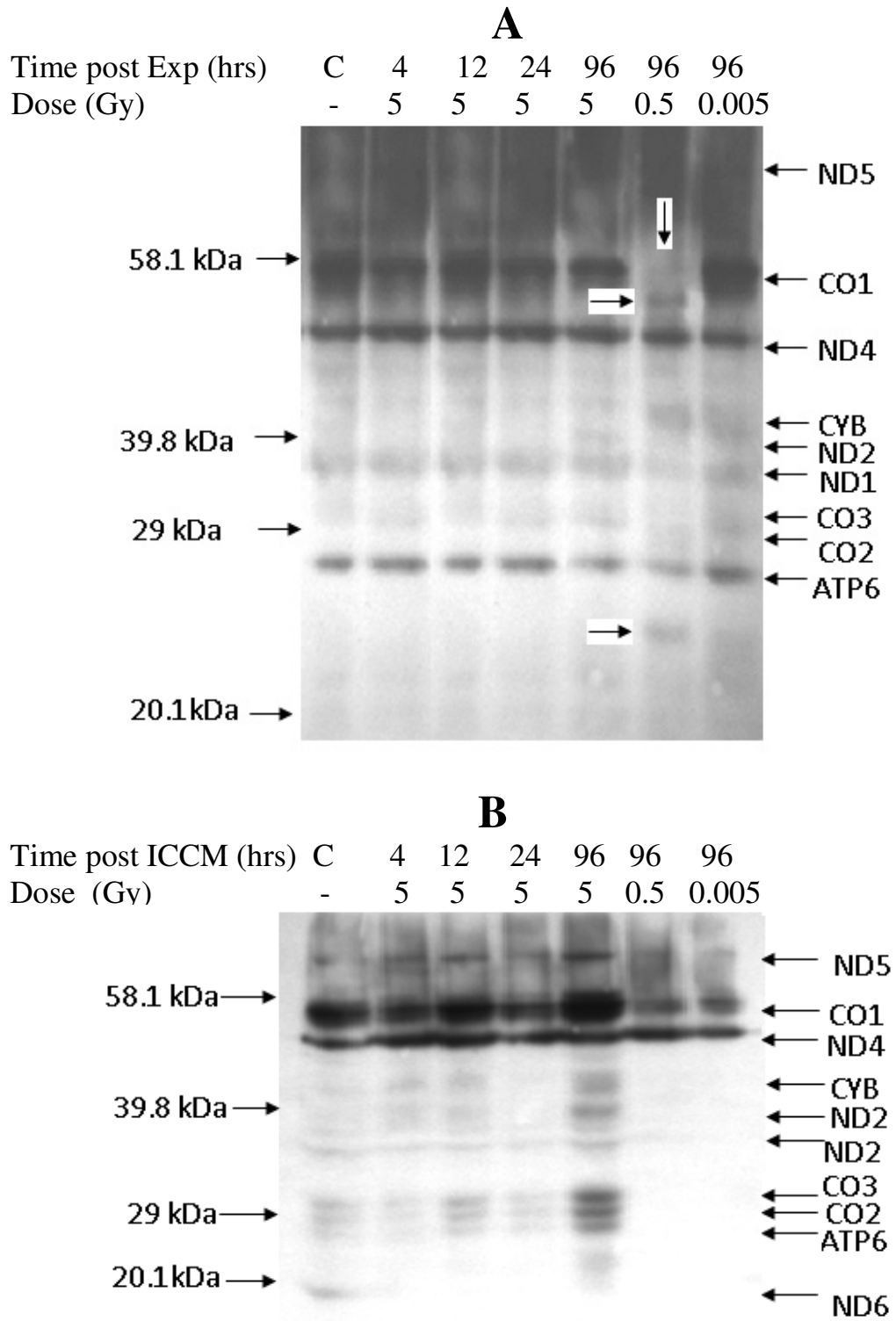


Figure 5

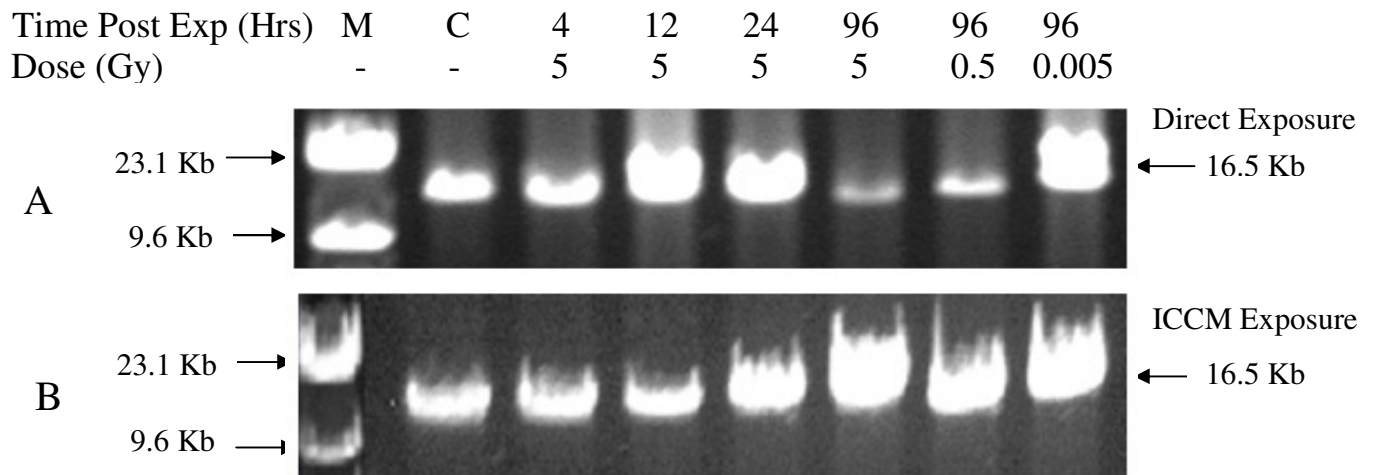
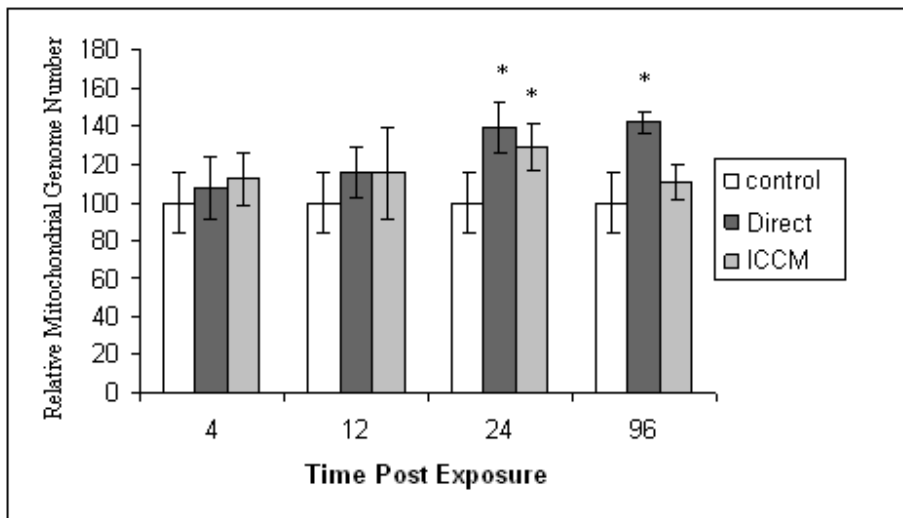


Figure 6

A



B

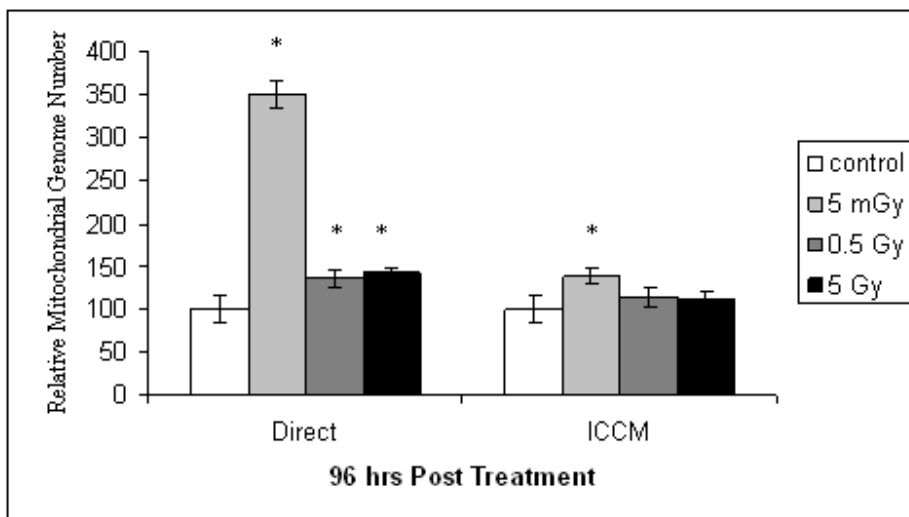


Figure 1: CHO-K1 OxPhos Enzyme Kinetics post direct radiation and ICCM.

CHO-K1 cells were harvested 4, 12, 24 and 96 hours post radiation and ICCM treatment. Enzyme kinetic assays were carried out to determine individual enzyme activity for (A) complex II-III, (B) complex IV and (C) complex V. * denotes significant when $p \leq 0.05$. Error bars indicate the standard error of the mean for triplicate experiments.

Figure 2: HPV-G OxPhos Enzyme Kinetics post direct irradiation and ICCM.

HPV-G cells were harvested 4, 12, 24 and 96 hours post radiation and ICCM treatment. Enzyme kinetic assays were carried out to determine individual enzyme activity for (A) complex II-III, (B) complex IV and (C) complex V. * denotes significant when $p \leq 0.05$. Error bars indicate the standard error of the mean for triplicate experiments.

Table 1: Summary of Ox Phos Enzyme Kinetics Data

Figures 1 and 2 are summarised here to better illustrate changes in OxPhos enzyme activity in CHO-K1 cells post direct irradiation (A), post ICCM (B) and in HPV-G cells post direct radiation (C) and ICCM (D). ↓ indicates a significant loss in activity compared to control, ↑ indicates a significant increase in activity compared to control and ↔ indicates no significant change in activity compared to control.

Figure 3: MtDNA-encoded protein synthesis in CHO-K1 cells.

MtDNA directed protein synthesis in CHO-K1 cells was analysed 4, 12, 24 and 96 hours after either (A) direct irradiation or (B) ICCM exposure. Only mtDNA-encoded polypeptides were

labelled with biotin and detected by conjugation, luminol conversion and autoradiography. Irregular banding patterns are highlighted with an arrow. Images are representative of triplicate experiments.

Figure 4: MtDNA-encoded protein synthesis in HPV-G cells.

MtDNA directed protein synthesis in HPV-G cells was analysed 4, 12, 24 and 96 hours after either (A) direct irradiation or (B) ICCM exposure. Only mtDNA-encoded polypeptides were labelled with biotin and detected by conjugation, luminol conversion and autoradiography. Irregular banding patterns are highlighted with an arrow. Images are representative of triplicate experiments.

Figure 5: Long Range PCR Analysis of HPV-G mtDNA

DNA was isolated post (A) direct irradiation and (B) ICCM treatment and equal amounts were added to each PCR reaction. Almost the entire genome was amplified and products were separated on a 0.8 % agarose. Images are representative of triplicate experiments. Equal DNA loading was confirmed by amplification of a region of conserved nuclear DNA (data not shown).

Figure 6: HPV-G Mitochondrial genome frequency analysis using Real-Time PCR.

Cells were harvested (A) 4, 12, 24 and 96 hours post 5Gy and 5Gy ICCM treatment and (B) 96 hours post 5mGy, 0.5Gy and 5Gy treatment. DNA was isolated and PCR was carried out with primers located in a conserved region of the mitochondrial genome and in the and in the β -actin gene in the nuclear genome (housekeeper). Relative frequency of

mtDNA was normalised against β -actin gene frequency and all data subsequently expressed as a percentage of control. Error bars indicate the standard error of the mean for triplicate experiments and * denotes statistical significance when $p \leq 0.05$.