NOTE TO USERS

Radiation response in cells derived from individuals with hereditary breast cancer

by

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ARE	Antioxidant response element
ATM	Ataxia-telangiectasia-mutated
BCI	Breast Cancer Information Core
BRCA1	Breast Cancer Susceptibility Gene 1
BRCA1:BARD1	BRCA1 associated ring domain 1
BRCT	BRCA1 carboxyl terminus
CEF	Cycholophosphamide, epirubicin, fluorouracil
DD	DNA damage
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl Sulfoxide
DSB	Double strand breaks
DTNB	5,5'-dithios(2-nitrobenzioc acid)
FBS	Fetal bovine serum
FSC	Forward scatter
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione/Glutathione disulphide
GST	Glutathione-S-transferases
HAAs	heterocyclic aromatic amines
HAT	Histone acetyl transferases
HDAC	Histone deacetylases
HR	Homologous recombination
IL-2	Interleukin 2
ITC	Isothiocyanate
Keap1	Kelch-like ECH-associated protein 1
LOH	Loss of heterozygosity
MDA	Malondialdehyde
MEF	Mouse embryonic fibroblast
MTT	tetrazole(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
NFAT	Nuclear factor of activated T-cells
NHEJ	Non-homologous end joining
NQO1	Quinone oxidoreductase
Nrf2	Nuclear erythroid2-related factor 2
PBS	Phosphate Buttered Saline
PHA	Phytohemagglutinin
ROS	Reactive oxygen species
SFN	Sulforaphane
siRNA	Small interfering RNA
SOD	Superoxide dismutase
SSA	Sulfosalicylic acid
SSC	Side scatter
TBS	Tris Buffered saline
TST	TBS containing 4% fetal bovine serum, 0.1% triton X-100

Abstract

Inherited germline mutations in the tumour suppressor gene BRCA1 (BReast CAncer susceptibility gene 1) can lead to an increased risk for various cancers. Evidence now suggests that epigenetic changes resulting in reduced or absent expression of BRCA1 are also important causative factors. BRCA1 has many known roles, such as DNA damage repair, cell cycle control and regulation of transcription. In addition, a recent study has identified yet another function for BRCA1: to protect cells from oxidative stress, a contributory factor in the development of many cancers, including breast cancer. BRCA1 is known to stimulate Nrf2, a transcription factor that regulates the expression of phase II enzymes, which are important in the detoxification of chemical compounds. Sulforaphane (SFN), an antioxidant found in cruciferous vegetables such as broccoli, has also been found to stimulate expression of phase II enzymes. The purpose of this study was to investigate the cellular consequences of exposure to ionizing radiation in primary blood lymphocytes from healthy individuals compared to cells from individuals with mutations in BRCA1. These responses include DNA damage, increased levels of oxidative stress and decreased cell viability. A secondary aspect of this study was to investigate the protective effects of SFN on lymphocytes exposed to ionizing radiation. The results suggest that there was a greater decrease in cell viability in lymphocytes derived from individuals carrying a germline BRCA1 mutation upon radiation exposure. It was also found that SFN did not protect cells against the damaging effects of ionizing radiation. This loss of viability correlated with an increase in DNA damage. However, these results are obtained from a limited sample size. The major accomplishments of this study were the development of techniques to study radiation responses using lymphocytes. To more completely investigate the questions of whether a germline mutation in BRCA1 significantly impacts the cellular radiation

response, and whether the dietary antioxidant SFN protects cells from the damaging impact of radiation, a larger sample size is needed in order for the results to be statistically significant.

1. Literature Review

1.1 Introduction

The background research that is published regarding the subject areas relevant to this project is presented. This includes information and statistics about breast cancer, as well as a discussion of general treatment strategies. The importance of the breast cancer susceptibility gene, BRCA1, and the consequences of loss of a functional BRCA1 allele will be explained. Next, the role that oxidative stress plays in the development of cancer will be reviewed. This is of particular interest because a major modality of cancer treatment is radiation therapy, which induces oxidative stress through the generation of cytotoxic levels of reactive oxygen species (ROS) in order to eradicate cancer cells. Lastly, the potential protective effects of a dietary antioxidant, sulforaphane (SFN) will be discussed.

1.2 Breast Cancer

Breast cancer is recognized as the most commonly diagnosed malignancy among women and affects 1.2 million women worldwide each year (reviewed in Fucito et al. 2008). In Canada, 1out of 8 women will be diagnosed with breast cancer in their lifetime. The breast cancer mortality rate has been declining steadily since 1993, but there are still many individuals and their families affected by this disease (NCIC 2007). Although genes that lead to an increased susceptibility to breast cancer have been discovered, germline mutations in these genes only account for a small percentage (<10%) of all breast cancer cases (Boulton 2006). Currently, evidence suggests that epigenetic changes resulting in reduced or absent expression of BRCA1 are important contributing factors in sporadic or non-hereditary breast cancer (Staff et al. 2003).

1.2.1 Etiology of breast cancer

Breast cancer is a multifactorial disease, meaning that there are many factors implicated in its development. These factors include: 1) hormone metabolism, as excess estrogen stimulates the growth of estrogen sensitive tumors (Messina et al. 2006); 2) environment, as exposure to carcinogens and exogenous hormones is implicated in tumour development (Chen et al. 2001; Lithgow and Covington 2005); 3) diet, as it has been suggested that fat intake leads to breast cancer and eating a low fat diet can reduce the risk of recurrent breast cancer (Anand et al. 2008); and 4) genetics, as a small percentage of breast cancers are familial or hereditary (Honrado et al. 2006). Germline mutations in the currently identified breast cancer susceptibility genes, BRCA1 and BRCA2, are only implicated in approximately 5-10% of all breast cancer cases (Daniel 2002).

1.2.2 Treatment Strategies

According to the *Clinical Practice Guidelines for the Care and Treatment of Breast Cancer* (CMAJ 2005), breast cancer treatment strategies typically consist of a combination of surgical removal of the tumour, chemotherapy and radiation therapy. Surgical removal is the first step and involves either a lumpectomy or a mastectomy. Both chemotherapy and radiation therapy induce cytotoxicity in the cancer cells to eradicate the tumour. Surgery can be followed by chemotherapy or radiation therapy, but treatment with both decreases the chance of recurrent disease and is often recommended. Treatment plans are prescribed based on the tumour histology and stage of cancer progression, the individual's age, and molecular markers identified in pathology specimens (CMAJ 2005).

Breast cancers that are caught early usually require a lumpectomy, which is removal of the tumour along with a "shell" of normal tissue to ensure the whole tumour has been removed,

followed by radiation therapy and chemotherapy. Breast tumours larger in size usually require a mastectomy (removal of the entire breast), and sometimes removal of surrounding lymph nodes (CMAJ 2005).

Molecular markers refer to whether the patient is positive for certain receptors, such as those for estrogen and progesterone and the HER2/neu receptor (James et al. 2007). Breast cancers positive for the hormone receptors have a better prognosis and are usually prescribed tamoxifen, which blocks estrogen from binding to its receptor, as excess estrogen can stimulate growth of the tumour (James et al. 2007). HER2/neu is an epidermal growth factor, and amplification of this receptor is associated with a poor prognosis (James et al. 2007). These breast cancers are treated with the antibody trastuzumab (Herceptin) which binds to the receptor and inhibits binding of the growth factor (James et al. 2007).

1.2.3 Summary of general breast cancer facts

Even though substantial progress has been made in the fight against breast cancer, there are still many things that remain unknown about this disease. Treatment strategies are currently based on individual and disease characteristics, such as age, progression of the disease and the presence of molecular markers. Individualization of cancer treatment is becoming more prevalent as taking the patient's individual characteristics into consideration, such as genetic status, generally results in favourable outcomes (Alpert and Haffty 2004). Further studies in this area could also shed light on cancer prevention strategies, including dietary changes that might include a more antioxidant-rich diet (Anand et al. 2008).

1.3 The role of BRCA1

The BRCA genes are classified as tumor suppressor genes, meaning that these genes are involved in protecting the genome from any sort of damage and are involved in the maintenance of genomic stability. This explains why those carrying a BRCA1 germline mutation have a 50-80% chance of developing cancers before the age of 70 (Deng and Wang 2003). Mutations in tumour suppressor genes, such as BRCA1, predispose individuals to cancer. Loss of the remaining functional allele, or loss of heterozygosity (LOH) is commonly observed in hereditary breast cancers (Staff et al. 2003). This follows Knudson's "two hit hypothesis" which was first developed in 1971 (Knudson 1971). The first "hit" or mutation refers to a germline mutation and the second hit refers to a somatic mutation that disrupts the expression or function of the tumour suppressor gene (Tucker and Friedman 2002). Without proper functioning or expression of BRCA1, the maintenance of genome integrity is lost, and the individual will likely develop cancer (Saal et al. 2008). Mutations in the BRCA1 tumour suppressor gene are not completely penetrant, meaning mutations in other tumour suppressor genes, such as p53 or PTEN, are necessary for the development of cancer (Saal et al. 2008). BRCA1 mutations appear to be tissue specific, as these mutations are notably associated with only breast and ovarian cancers (Staff et al. 2003); however, there is evidence to suggest that BRCA1 mutation may play a role in other cancers, such as prostate, lung and pancreatic cancer (Rosen et al. 2003).

The BRCA1 gene is located on chromosome 17 and encodes a 220 kDa nuclear protein, and its function is not completely understood, even though it has been extensively studied since it was first identified in the mid 1990's. BRCA1 participates in many cellular processes including transcriptional regulation, apoptosis, cell cycle regulation, and DNA repair (Daniel 2002). Based on its many protein interactions and cellular functions, it is clear that BRCA1 has

a broad role in the cellular response to DNA damage (Figure 1) (MacLachlan et al. 2000). The role of BRCA1 in transcriptional regulation has been revealed in several studies that showed its direct interaction with other transcriptional activators and repressors, and that it is phosphorylated in response to various DNA damaging agents, leading to regulatory changes in the expression of various target genes (Rosen et al. 2006). Because BRCA1 plays a role in DNA repair and transcriptional regulation and functions at checkpoints within the cell cycle, it is evident that germline mutations in the BRCA1 gene would predispose individuals to genetic instability, and hasten the initiation and progression of cancer.

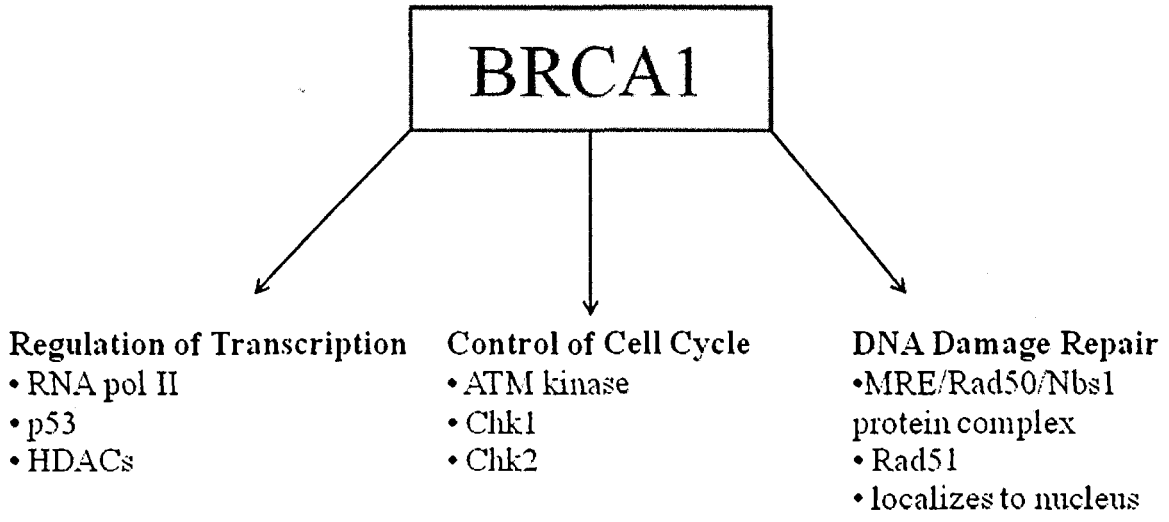


Figure 1: BRCA1 interactions that are highlighted in the text. Transcriptional regulation, cell cycle control, and DNA damage repair are mentioned.

1.3.1 Regulation of transcription

Although BRCA1 is not a sequence-specific DNA-binding transcription factor, studies have shown that it can regulate gene transcription. BRCA1 has been found to interact with basal transcription machinery, such as RNA polymerase II (RNA pol II). The BRCA1:BARD1 (BRCA associated ring domain 1) complex within the RNA pol II enzyme becomes activated at sites of DNA damage and stops transcription. Once transcription is interrupted, the transcription

machinery is ubiquitinated and degraded (Rosen et al. 2006). It is suggested that the BRCA1/BARD1 complex activates the ubiquitin ligase (Venkitaraman 2002). BRCA1 will also interact with specific transcription factors and can either stimulate or inhibit transcription. The interaction of BRCA1 with transcriptional regulators can control the expression of genes involved in many cell processes, such as genes involved in apoptosis and DNA repair. An example of this is with the interaction of BRCA1 with p53. p53 is a very important nuclear transcription factor, and another tumour suppressor gene, and it is found to be mutated in >50% of all cancers (Bhana et al. 2008). BRCA1/p53 interaction specifically induces the expression of genes involved in DNA repair and cell cycle arrest; however, if p53 were to bind to another protein, apoptosis could occur. The coupling of these two tumor suppressor genes appears to decide the fate of a cell in a situation involving extensive DNA damage (Rosen et al. 2006).

The BRCA1 carboxyl terminus (BRCT domain) has been found to associate with histone deacetylases (HDAC) and histone acetyl transferases (HATs) (Oishi et al. 2006; Yarden and Brody 1999). HDACs remove acetyl groups from histones, whereas HATs function to attach these groups. When acetyl groups are attached to histone proteins, DNA is accessible to the proteins responsible for activating transcription, such as RNA polymerase II. The BRCT domain interacts with HDAC1 and 2 to inhibit transcription of various genes; however, when the BRCT domain interacts with HATs, gene transcription is activated (Park et al. 2008). Therefore, without interaction of BRCA1 with the HDACs, continuous transcription of genes that are not needed can occur; and conversely, without interaction with the HATs, transcription of needed genes, may not happen.

1.3.2 Control of the cell cycle

The cell cycle is strictly regulated by checkpoints to ensure that the cell does not progress through the cycle if it is compromised in any way. BRCA1 is involved at all checkpoints of the cell cycle (MacLachlan et al. 2000).

The BRCA1:BARD1 complex has also been shown to initiate the ATM (ataxia-telangiectasia-mutated) phosphorylation of p53 at Ser-15 following ionizing radiation. Phosphorylated p53 mediates activation of p21, a cyclin-dependent kinase inhibitor, which will induce G_1/S arrest (Deng and Wang 2003).

In addition, BRCA1 plays a role at the S phase cell cycle checkpoint. ATM will phosphorylate a specific serine residue on BRCA1 (Ser-1387) to induce intra-S-phase arrest. Failure to halt progression of the cell cycle at this point leads to continuous DNA synthesis of unrepaired strands (Gudmundsdottir and Ashworth 2006).

At the G_2/M checkpoint, there are three important BRCA1 interactions. The first of these is with a known regulator of the G_2/M checkpoint, Chk1. BRCA1 regulates the expression, phosphorylation and localization of this protein. The second is another interaction between BRCA1 and the ATM kinase. At this checkpoint, ATM phosphorylates BRCA1 at another serine residue (Ser-1423), in order to halt progression through the cell cycle in response to DNA damage (Gudmundsdottir and Ashworth 2006). The last major interaction of BRCA1 at this checkpoint is with Chk2. This protein is an immediate phosphorylation target of the ATM kinase. Chk2 then phosphorylates Ser-988 on the BRCA1 protein. At this checkpoint, BRCA1 has been shown through knockout studies to be necessary for modulation of the DNA damage response and tumor repression (Deng and Wang 2003).

The importance of BRCA1 throughout the cell cycle is evident. Failure to halt progression of the cell cycle when DNA is damaged leads to continued synthesis of damaged DNA and unchecked proliferation of mutated cells. If allowed to continue, these damaged cells can potentially develop into a tumor.

1.3.3 DNA damage repair

BRCA1 plays a very important role in the cellular response to DNA damage. It is activated when damage occurs and will either bind to the site and recruit other DNA repair enzymes, or bind with other proteins as part of a DNA repair complex.

A DNA damaging agent, such as ionizing radiation, will induce DNA double strand breaks (DSBs) through the induction of oxidative stress. This type of DNA damage, if left unrepaired or if repaired incorrectly, is mutagenic (Gudmundsdottir and Ashworth 2006). Studies have shown that BRCA1-deficient cells repair this type of damage through non-conservative DNA repair mechanisms, such as single strand annealing (SSA) or non-homologous end-joining (NHEJ), leading to genetic instability and further mutations (Turner et al. 2005). BRCA1 is necessary for the conservative DNA repair process of homologous recombination (HR) (Turner et al. 2005). At the site of a DSB, the histone protein H2A.X becomes phosphorylated (γ-H2A.X), and studies have shown that once this occurs, BRCA1 will localize to the nucleus (Daniel 2002). BRCA1 has also been found to interact with the MRE/Rad50/Nbs1 protein complex, which is known to localize to the nucleus and participate in DSB repair. Using immunochemical techniques, it was shown that this complex co-migrates to the nucleus to the site of the DSB (Venkitaraman 2002).

1.3.4 The role of BRCA1 in sporadic cancers

The role of BRCA1 in sporadic breast cancers remains unknown. Some studies suggest that BRCA1 can become epigenetically modified through promoter methylation (Esteller et al. 2000). Although it remains unclear what the exact role of BRCA1 is in sporadic breast cancers, it is evident that at some point during the progression of the disease, it can become inactivated (Wilson et al. 1999). This has been shown through immunohistological staining of high grade tumors where BRCA1 is undetectable, but can be detected in the adjacent healthy tissues (Wilson et al. 1999). Lack of BRCA1 expression or altered localization of the protein is also associated with advanced lymph node stage, larger tumour size, vascular invasion, and negative hormone receptor expression (Rakha et al. 2008). In sporadic cancers, BRCA1 expression can be lost through BRCA1 promoter methylation, but that is not always the cause and other specific mechanisms are unknown (Esteller et al. 2000). Further research needs to be done to uncover other possible mechanisms through which expression of BRCA1 is lost in non-hereditary breast cancers.

1.3.5 Summary of the role of BRCA1

Loss of BRCA1 expression leads to a loss of genomic stability through many mechanisms including: interference with transcription of certain genes, loss of cell cycle control, and loss of specific DNA repair mechanisms. Altered DNA repair capacity and the associated cellular changes in individuals with decreased or absent BRCA1 expression will predispose such an individual to the development of cancer. The role of BRCA1 is sporadic breast tumours remains unknown; however, the expression or function of this gene is absent in high-grade tumours.

1.4 Radiation therapy and the induction of oxidative stress

Oxidative stress refers to the total burden of potentially harmful reactive oxygen species (ROS) that are present in cells as a consequence of routine cellular oxidative metabolism (Valko et al. 2006). In healthy cells, ROS occur at low to moderate levels and have beneficial effects, such as protecting cells from infectious agents and inducing the mitogenic response under appropriate circumstances; however, an excess of ROS plays an important role in disease conditions, including breast cancer (Valko et al. 2007). Although the generation of ROS can lead to mutations that could result in cancers, this property is also exploited in both radiation therapy and many types of chemotherapy to induce cytoxicity in cancer cells, leading to their eradication.

1.4.1 Reactive oxygen species (ROS)

ROS are made up hydrogen peroxide and oxygen-containing free radicals. Free radicals are molecules which contain unpaired electrons such as the superoxide anion (O_2^-), the peroxyl radical (ROO^-) and the hydroxyl radical (OH^-). The superoxide anion is produced first and is considered the "primary" ROS. The superoxide radical will interact with other molecules to produce "secondary" ROS, such as hydrogen peroxide, which can be broken down further into the damage inducing hydroxyl radical (Fridovich 1986). The human cell is exposed to thousands of oxidative injuries per day (Dizdaroglu et al. 2002).

Endogenous sources of ROS include the mitochondrial respiratory chain, cytochrome P_{450} metabolism, peroxisomes, and inflammatory cell activation of the immune system. Mitochondria produce the largest physiological source of the superoxide radical which is produced at approximately 2-3 nM per minute per milligram of protein (Inoue et al. 2003). Cytochrome P_{450} metabolism produces both hydrogen peroxide and the superoxide radical (Inoue

et al. 2003). Peroxisomes, which are predominantly found in the liver, produce hydrogen peroxide under physiological conditions (Gupta et al. 1997). The immune system reacts to an infection through the release of ROS and cytokines to kill the invading infectious agent (Lithgow and Covington 2005).

Exogenous sources of ROS include environmental agents such as cigarette smoke and environmental pollutants (Lithgow and Covington 2005). Particles of trace metals in the air such as arsenic, vanadium, and chromium, are also considered to be environmental pollutants which induce and contribute to the development of disease conditions, including cancer (Chen et al. 2001). Another source of exogenous ROS is ionizing radiation, which is a major modality of cancer treatment.

1.4.2 Damaging effects on the cell and the disruption of signaling pathways

Reactive oxygen species can attack all major cellular components, including DNA, lipids, and protein. The hydroxyl radical is short lived, and therefore reacts close to its site of formation. When produced near DNA, the hydroxyl radical is known to interact with both the DNA bases and the sugar-phosphate backbone, causing single or double stranded breaks, DNA cross-links and oxidative base modifications (Valko et al. 2006). Oxidative damage to the genetic material can cause replication errors, disrupt signalling pathways, and cause genomic instability, which are all associated with carcinogenesis (Dizdaroglu et al. 2002).

Lipid peroxidation is another form of oxidative damage occurring through the action of the free radicals on poly-unsaturated fatty acids (Valko et al. 2006). Lipid peroxidation end products include malondialdehyde (MDA) and hydroxynonenal (HNE). MDA has been found to be mutagenic and carcinogenic in rats (Valko et al. 2006). MDA can interact with DNA bases to form DNA adducts. Of particular interest, MDA will interact with guanine (G) to form the

MDA-DNA adduct, M_1G, which has been detected in human breast tissue (Wang et al. 1996). HNE is weakly mutagenic, but it has a powerful effect on signal transduction pathways, which will impact cell phenotype (Valko et al. 2006). It has been shown that increased levels of HNE coincide with a loss of mismatch repair enzymes in breast cancer tissues (Karihtala et al. 2006).

Protein damage is usually repairable and non-lethal (Stadtman 2004). The side chains of amino acids are susceptible to oxidative damage through ionizing radiation and endogenous ROS (Stadtman 2004). The modification of proteins involved in signal transduction, such as protein kinases or phosphatases, by ROS can have detrimental effects on the regulation of gene expression.

The end targets of all signaling pathways are transcription factors, which induce the expression of specific genes. Specific transcription factors that are activated via ROS signalling are involved in the transcription of genes which aid in cell proliferation and survival. These include AP-1, NF-κB, p53, and NFATs (Nuclear factor of activated T-cells). Proliferation and oncogenic transformation in breast cancer cells can be induced by activation of AP-1 through growth factors such as estrogen, EGF, TGFα and IGFs (Shen et al. 2008). NF-κB blocks apoptosis, promotes cell proliferation, and is activated by the inflammatory cytokines, TNF-α and IL-1 (Bubici et al. 2004). It regulates progression through a cell-cycle checkpoint and if the cell is damaged, it can initiate apoptosis. Studies have found that oxidative damage can hinder the function of p53, either by inhibiting it's relocation to the nucleus or through post-translational modification (Godon et al. 2005; Renzing et al. 1996). The NFAT family of transcription factors regulate cytokine formation and angiogenesis, and have also been found to interact with AP-1 and NF-κB (Valko et al. 2006).

1.4.3 Radiation therapy: the purposeful induction of ROS as a cancer treatment

Radiation therapy is considered a major modality of cancer treatment and kills the tumour cells by inducing cytotoxic levels of ROS. DNA double strand breaks are the most dangerous lesion that can occur in DNA in response to ionizing radiation. The induction of multiple double strand breaks overloads a cell's DNA repair capacity, resulting in cell death (Wang et al. 2001). Radiation can induce these double stranded breaks either directly or indirectly (Figure 2).

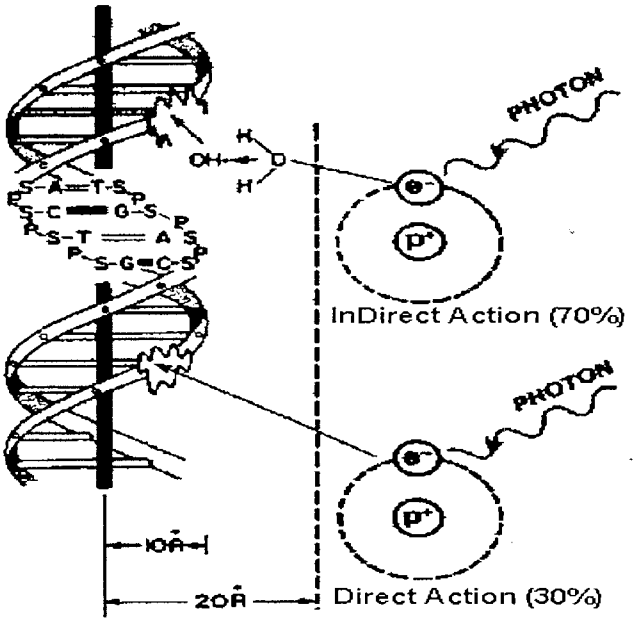


Figure 2: The indirect and direct action of radiation. From Hall, Eric J. *Radiobiology for the Radiologist*, 5th ed. Philadephia PA: Lippincott Williams & Wilkins, 2000.

Approximately 30% of damage to DNA occurs directly, when the photon interacts directly with the DNA strands to induce damage. The majority of the damage, approximately 70%, occurs indirectly. In this case, the photon interacts with others molecule within the cell, usually water, to produce the hydroxyl radical, which will then make contact with DNA and induce damage (Hall 2000). As previously mentioned, when the hydroxyl radical is produced near DNA, it will interact with both the DNA bases and the sugar-phosphate backbone, inducing double strand breaks (Valko et al. 2006). Radiation therapy can inadvertently damage

surrounding healthy tissue through leakage or scatter of the radiation during delivery (Hall 2000), and if not repaired properly, DNA double strand breaks can lead to cell death or the induction of mutations and gene translocations which may eventually lead to the development of therapy-induced cancer (Bau et al. 2006).

1.4.4 Treatment strategies and clinical management of BRCA1 mutation carriers

The clinical management of breast cancer patients with BRCA1 mutations remains controversial. A BRCA1 germline mutation would result in altered DNA repair capacity and associated cellular changes in the affected individual. Therefore, treatment with radiation therapy would make this individual more susceptible to radiation-induced complications in normal tissue. These complications include an increase in the incidence of a secondary malignancy (Alpert and Haffty 2004). Strategies to prevent secondary malignancies include radical surgery such as, prophylactic mastectomy and oophorectomy (removal of the ovaries). Both of these surgeries have been found to reduce the incidence of secondary breast and ovarian cancers in individuals carrying a germline BRCA1 mutation (Alpert and Haffty 2004). Tamoxifen is also usually recommended for patients with estrogen receptor-positive tumours as it prevents estrogen from stimulating the growth of tumours (Alpert and Haffty 2004).

Currently, research is being focused towards further individualizing cancer treatment strategies in order to maximize therapeutic benefit while minimizing the risk of therapy-induced cancer and later complications (Travis et al. 2006). Studies suggest that factors such as diet, age, and genetic background should be taken into consideration when devising a treatment strategy (Dumitrescu and Cotarla 2005). This present study on cells from individuals with BRCA1 mutations may lead to a better understanding of how radiation therapy induces cytotoxicity in

cancer cells, and on the recurrent cancers that could result from secondary mutations in surviving cells or in surrounding tissues.

1.5 Antioxidant defense systems of the cell

Cells contain antioxidants, both enzymatic and non-enzymatic, that will neutralize ROS. Enzymatic antioxidants include superoxide dismutase, catalase and glutathione peroxidase . Non-enzymatic antioxidants include, among many, vitamin C, vitamin E, thiol antioxidants, such as glutathione, and natural flavonoids (Valko et al. 2007).

1.5.1 The enzymatic and non-enzymatic antioxidants

Superoxide dismutase (SOD) is one of the most efficient enzymatic antioxidants. SOD will break down the superoxide anion to form hydrogen peroxide and oxygen. There are three classes of human SODs: mitochondrial (Mn-SOD), cytosolic (Cu, Zn-SOD), and extracellular (Landis and Tower 2005). SOD will work in conjunction with other enzymes such as catalase and glutathione peroxidase (GPx). Both catalase and GPx will break down hydrogen peroxide. Catalase can be found in the peroxisome and degrades hydrogen peroxide to water and molecular oxygen. GPx also decomposes hydrogen peroxides into water while oxidizing glutathione (GSSG). GPx uses the non-enzymatic antioxidant glutathione (GSH) as a substrate.

As mentioned, the non-enzymatic antioxidants include vitamin C, vitamin E, thiol antioxidants, such as glutathione, and natural flavonoids. The thiol antioxidant, GSH, is a tripeptide of γ-glutamate, cysteine and glycine and it is one of the major reductants found in eukaryotic cells (McEligot et al. 2005). GSH exists predominantly in the reduced form in the cytosol, mitochondria, and nucleus, and therefore protects the cell from the oxidants produced during normal metabolism (Valko et al. 2007). The antioxidant capacity of this tripeptide comes from the sulphur moiety on the cysteine residue, which will easily give up an electron and

become oxidized (Valko et al. 2006). In the presence of ROS, and through interaction with other enzymes such as GPx, GSH is converted to the oxidized form, glutathione disulphide (GSSG). Glutathione-S-transferases (GSTs) initiate the reaction of GSH with electrophiles and physiological metabolites, such as estrogen, to begin detoxification (Wu et al. 2004). Glutathione reductase is responsible, along with NAD(P)H, for the conversion of GSSG back to GSH (Valko et al. 2006). One of the major indicators of the redox status of a cell is the GSH/GSSG ratio system. An increase in redox ratio indicates a decrease in oxidative stress. The redox ratio decreases as the concentration of GSSG increases (Wu et al. 2004).

1.5.2 The antioxidant response element (ARE)

The antioxidant response element (ARE) is a specific DNA sequence found near the promoter that mediates the transcriptional activation of many antioxidant genes. These genes include enzymes involved in glutathione biosynthesis, as well as a class of detoxifying enzymes, called Phase II enzymes (Nguyen et al. 2003). Phase II enzymes include GST, NAD(P)H quinone oxidoreductase (NQO1) and glutathione reductase (GR) (Jeong et al. 2006). As previously mentioned, these enzymes are important in detoxifying processes. GST initiates the reaction of GSH with electrophiles to begin the detoxifying process. NQO1 can reduce both NAD(P)H and NADP so that NAD(P)H can act as a co-factor with GR and reduce GSSG back to GSH (Jeong et al. 2006; Valko et al. 2006). Activation of these enzymes protects the cells from the toxic and neoplastic effects of many carcinogens, and therefore inhibits the initiation of carcinogenesis. This occurs through interactions with endogenous antioxidants, such as GSH, that convert toxic substances to less reactive materials that increases their solubility and excretion, as well as by increasing the cellular antioxidant capacity through generation of more antioxidant molecules like GSH (Jeong et al. 2006).

The transcription factor responsible for the activation of the ARE is Nrf2 (nuclear-erythroid 2-related factor 2) (Nguyen et al. 2003). Studies also demonstrated that Nrf2 specifically induces the transcription of GR, GPX, and GSTs, and all of these enzymes use GSH as a substrate to detoxify electrophiles. In general, the expression of genes that are involved in detoxification, antioxidant status, cytoprotection, and repair indicates an important role for the activation of Nrf2 and the ARE in cancer prevention (Thimmulappa et al. 2002).

1.5.3 Potential role of BRCA1 in the activation of the ARE

A study done by Bae and colleagues (2004) is an important foundation for this study. They extensively investigated the effects of overexpression and underexpression of BRCA1 in various cell lines (Bae et al. 2004). First, they did microarray analyses and found that cells expressing wild type BRCA1 up-regulated genes involved in transcription, stress responses, DNA replication and repair, signal transduction, metabolism, differentiation, and RNA and protein processing. These genes included phase II enzymes: glutathione S-transferases (GSTs), glutathione peroxidase 3 (GPx3), and oxidoreductases (NQO1). Conversely, BRCA1-mutant cells downregulated the expression of genes involved in transcription, stress response, cell cycle regulation, DNA replication and repair, and signal transduction. Also, there was no significant expression of the phase II enzymes in these cells (Bae et al. 2004).

To test that BRCA1 protection against oxidants is mediated by regulation of Nrf2, Bae and colleagues used transient transfection assays using a Nrf2 responsive reporter that is driven by the ARE. They found that increasing the amount of wtBRCA1 plasmid increased the activity of the reporter gene. They also found that cells treated with siRNA that blocks transcription of BRCA1 were more sensitive to hydrogen peroxide treatment. Lastly, they determined structural requirements for interaction between BRCA1 and Nrf2 using a series of vectors encoding

truncated or mutant BRCA1 proteins. A point mutation in the NH_2-terminal RING domain or an NH_2-terminal truncation hindered the ability of BRCA1 to stimulate transcriptional activation of phase II enzymes (Bae et al. 2004).

This newly identified activity of BRCA1 is of great interest. BRCA1 up-regulates the expression of multiple antioxidant genes, indicating that the role of BRCA1 in protection against the cytotoxic effects of ROS involves not only specific DNA repair mechanisms, but that BRCA1 plays a role in transcriptional regulation of the cellular antioxidant response. Furthermore, Abbott and colleagues (1999) studied the effects of ionizing radiation on cells homozygous for BRCA1 mutation, and found that these cells were significantly more radiosensitive than BRCA1 competent cells (Abbott et al. 1999). This was due to the lack of transcription coupled repair found in BRCA1 deficient cells, providing additional evidence that BRCA1 interaction with componenets of the transcription machinery, such as RNA pol II, are physiologically relevant (Abbott et al. 1999).

1.5.4 Possible protective mechanism of sulforaphane

Sulforaphane (SFN) is an isothiocyanate (ITC), and its structure consists of a large planar molecule containing a thiol group. Sulforaphane is an antioxidant found in dark green cruciferous vegetables such as broccoli. As previously mentioned, epidemiologic studies have found that the consumption of cruciferous vegetables is associated with a decrease in cancer risk (Dumitrescu and Cotarla 2005). SFN has both cytotoxic and antioxidant properties, depending on the cell type and the disease condition studied, however the cytotoxic effects are discussed here. Studies have shown that SFN has been found to induce cell cycle arrest and apoptosis in human T-cell leukemia cells, and apoptosis in human osteosarcoma cells (Fimognari et al. 2002; Matsui et al. 2006). SFN has been found to inhibit histone deacetylases (HDACs) in prostate

cancer cells (Myzak et al. 2006). Antioxidant properties include activation of the ARE through regulation of the transcription factor Nrf2 to induce phase II enzyme expression (Figure 3).

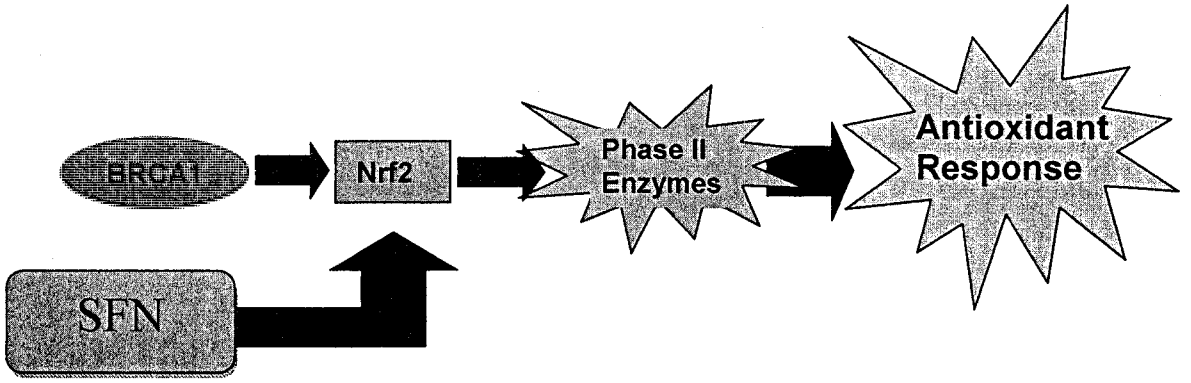


Figure 3: Mechanism and impacts of SFN.

Nrf2 activity is regulated through interaction with Keap1 (Kelch-like-ECH-associated protein 1). When bound to Keap1, Nrf2 remains inactive and stays in the cytoplasm, where it is degraded (Jeong et al. 2006). Keap1 is a cysteine-rich protein, and certain residues are sensitive to the presence of oxidants and release Nrf2 in the presence of oxidants (Jeong et al. 2006). However, being a cysteine-rich protein, Keap1 is vulnerable to modification by molecules containing a thiol group, such as SFN (Nguyen et al. 2003). Upon interaction of SFN with Keap1, Nrf2 is released and is now free to localize to the nucleus and bind to the ARE and induce phase II enzyme expression (Myzak and Dashwood 2006).

SFN could offer protection to cells against a build-up of ROS. In the future, the individualization of cancer treatment could place further importance on the diet. Dietary inducers of phase II enzyme expression may induce a multitude of antioxidant enzymes and increase the overall tissue antioxidant potential without the risk of converting them into oxidants, which is part of a cancer prevention strategy.

1.5.5 Summary of the role of BRCA1 and the ARE

BRCA1 is reported to stimulate the activity of Nrf2 and therefore plays a direct role in resistance to oxidative stress. BRCA1 is also involved in the up-regulation of multiple antioxidant genes, indicating that the role of BRCA1 in protection against the cytotoxic effects of ROS involves not only specific DNA repair mechanisms, but that BRCA1 also plays a role in transcriptional regulation of the cellular antioxidant response.

1.6 Conclusions

The background relating to this project has been explained in detail. This includes the causes of breast cancer, with a specific focus on the predisposition of individuals affected with a BRCA1 germline mutation. The importance of BRCA1 in the maintenance of genomic stability and how the functions of BRCA1 can dissipate the harmful effects of an excess of reactive oxygen species produced through ionizing radiation were described. Lack of functional BRCA1 will predispose an individual to the development of cancer, and perhaps, to the development of secondary malignancies. The potential of BRCA1 to act as an inducer of antioxidants through the induction of the transcription factor Nrf2 has been discussed. The antioxidant mechanism of SFN through the activation of the transcription factor Nrf2 was also described.

The methods used in this study will investigate the levels of DNA damage, levels of oxidative stress and viability of lymphocytes obtained from individuals with BRCA1 germline mutations and healthy controls, as well as the effects of SFN on these readouts.

2. Project summary

2.1 Purpose and hypotheses

Breast cancer is the most frequently diagnosed cancer in women, and the rates are three times higher in western countries than in developing countries. Certain risk factors are known to play a role in breast cancer development and these include, but are not limited to, genetics, hormones, diet and environmental factors. Epidemiologic studies have shown that consumption of cruciferous vegetables is strongly associated with a decrease in risk of cancer. BRCA1 (Breast Cancer susceptibility gene 1) was the first gene to be identified with an increased risk of breast cancers, and several more have since been added to this expanding list. BRCA1 plays an integral role in the maintenance of genomic stability and protection against DNA damage. This gene is important in the development of breast cancer and may also be important in the response to therapy used to manage the disease, such as radiation therapy. Radiation therapy exploits the use of ionizing radiation to induce sufficient oxidative stress in tumour cells to cause cytotoxicity, therefore killing the tumour. However, it is difficult to completely avoid the healthy surrounding cells, which may be affected when exposed to these high doses of radiation. Altered DNA repair capacity and associated cellular changes in individuals with decreased or absent BRCA1 function may make these individuals more susceptible to radiation-induced complications in normal tissue or the development of secondary malignancies and recurrent disease.

The purpose of this study was to investigate the cellular response of primary blood cells from healthy individuals compared to cells from individuals with mutations in BRCA1 following exposure to ionizing radiation. These responses include levels of DNA damage, levels of oxidative stress and cell viability.

A secondary aspect of this study is to investigate the effects of sulforaphane (SFN), a dietary antioxidant derived from cruciferous vegetables, in lymphocytes from individuals with breast cancer.

The specific aims of this study were:

1. To measure the basal and radiation-induced levels of DNA damage, cell death and change in cellular redox state between lymphocytes from individuals with a known BRCA1 mutation and individuals with no BRCA1 mutation.
2. To determine if pretreatment with SFN modulates the above mentioned response induced by ionizing radiation in lymphocytes.

Hypotheses:

1. Radiation damage will be greater in lymphocytes from individuals with BRCA1 mutations due to lack of DNA repair capacity.
2. The addition of SFN will protect the lymphocytes against ionizing radiation induced damage.

2.2 Broader scope of research project

The main focus of this thesis was to analyze the differences in levels of oxidative stress and DNA damage between healthy controls and two individuals with an identified BRCA1 mutation. However, this thesis is part of a larger research project also involving individuals with sporadic breast cancer, as well as those afflicted with BRCA1-associated familial breast cancer. There are also other cellular characteristics that need to be investigated, including BRCA1 protein expression levels, as well as BRCA1 mRNA levels (Figure 4).

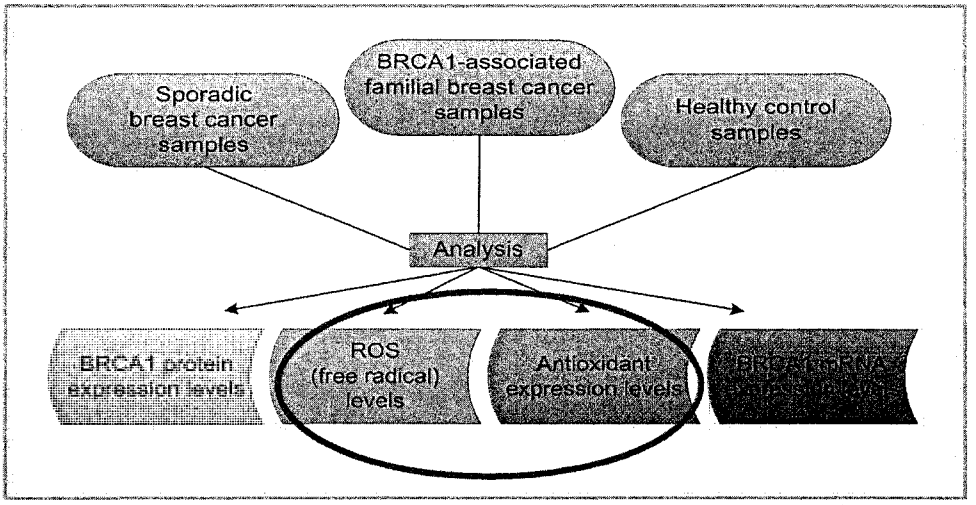


Figure 4: This schematic illustrates the broader scope of this research project, which includes samples from individuals with sporadic breast cancer and other cellular readouts such as BRCA1 protein expression and levels of BRCA1 mRNA, as well as ROS and antioxidant expression levels, which are the specific interests of this project.

The involvement of BRCA1 in sporadic breast cancers is poorly understood, however studies have shown expression of BRCA1 in high-grade sporadic tumours is absent. Also, epigenetic modification of BRCA1 contributes to the development of a certain subset of breast cancers.

2.3 Ethics approval and special collaborations

As this study required the use of human blood samples, the project was submitted to be reviewed for ethics approval. A request was put forth for 20 ml of blood to be drawn from each individual participating in the study. Ethics approval was sought through the Thunder Bay Regional Health Sciences Centre (TBRHSC) Research Ethics Team (Appendix 1 and 2). The request was granted in October of 2006. This project is also part of a continuing collaboration with the Northwestern Ontario Regional Genetics Program (Appendix 3) in order to find individuals identified as having a mutation in the BRCA1 gene and the Radiation Oncology Department at TBRHSC for the recruitment of individuals with sporadic breast cancer.

2.4 Significance

There is recent increased interest in the individualization of cancer treatment, as it is becoming more and more apparent that an individuals genetic makeup has a major impact on outcomes. To date, studies on BRCA1 have been limited to established cell lines that have relatively high cell division rates and therefore require greater metabolism. This study is novel because to date, there have been no studies that correlate BRCA1 activity with cellular response to radiation therapy using patient samples which have much lower division rates and presumably lower metabolic activity than immortalized breast cancer cell lines. We used peripheral blood lymphocytes from individuals with BRCA1-associated familial breast cancer and compared levels of DNA damage and redox potential resulting from radiation exposure. Lastly, the effects of a dietary antioxidant, SFN, in lymphocytes from individuals with breast cancer were investigated. This will provide data as to whether dietary antioxidants can protect against the endogenous generation of excess reactive oxygen species and potentially reduce the risk of cancer, even in individuals that have a genetic predisposition to the disease.

3. Materials and Methods

3.1 Introduction

This study was initiated with experiments using the breast cancer cell line, MCF-7. The MCF-7 cell line is a breast cancer cell line that is commonly used in cancer research. Cell lines were used to establish the techniques that would be potentially applied to primary cells. Primary cells were isolated from healthy volunteer controls for the final optimization of techniques. Once techniques were optimized, individuals were identified through the Northwestern Ontario Regional Genetics Program based on their BRCA1 mutation status and recruited to participate in this study. The study samples were divided into four groups and various techniques were used to detect cell viability, cellular redox potential, and DNA damage (Figure 5). The optimization and setup for the delivery of radiation will also be described.

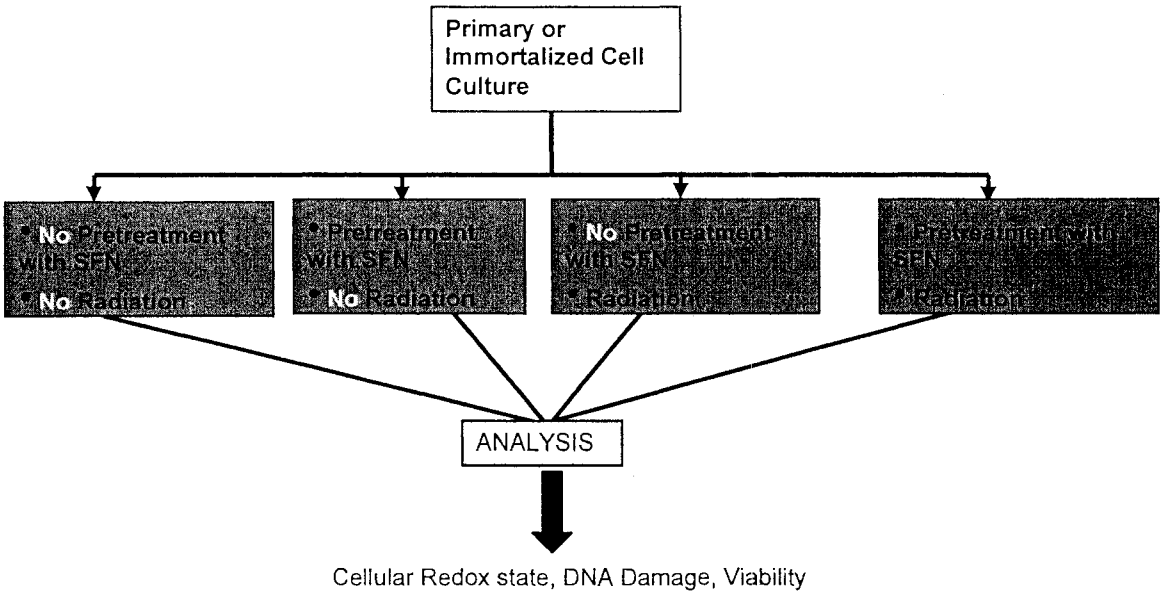


Figure 5: A schematic depicting the four study groups that each sample will be divided into, and the outcomes that will be investigated in this study.

3.2 Cell culture conditions

3.2.1 Propagation of MCF-7 cells

This study used the MCF-7 cell line, which was kindly provided by Dr. M.L. Tassotto. This cell line was maintained in a humidified atmosphere at 37°C and 5% CO_2.

MCF-7 cells have no known BRCA1 mutation and are epithelial adenocarcinoma cells derived from a pleural effusion metastasis from a primary tumour in the mammary gland of a 69-year-old female. They were originally obtained from the American Type Culture Collection. They were propagated in Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 10% Fetal Bovine Serum (FBS) and antibiotic/antimycotic (100 units/ml penicillin G Sodium, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B). Cell cultures were maintained in 100mm tissue culture dishes. Medium was renewed every two or three days and cells were subcultured upon reaching 85% confluence. To subculture, culture medium was aspirated and the cells were rinsed twice with PBS (Dulbecco's phosphate buffered saline, Sigma) to remove any traces of serum. A volume of 1.5 ml of 0.05% trypsin-EDTA solution (Gibco) was added and incubated for approximately three minutes at 37°C. Once cells had detached from the surface of the plate, medium was added to inactivate the trypsin. The cell suspension was divided between 100 mm tissue culture dishes as needed.

3.2.2 Isolation of human peripheral blood lymphocytes

Blood samples were obtained by certified phlebotomists in the Ambulatory Care department at Thunder Bay Regional Health Sciences Centre (Appendix 4). Whole blood obtained from the large vein of the forearm was diluted at a 1:1 ratio with PBS, and 8 ml of diluted blood was layered over 6ml Ficoll-Paque PLUS in a 15 ml Falcon tube and centrifuged at 800×*g* for 40 minutes at 18-20°C with the brake off (Figure 6). The lymphocyte layer was

collected and resuspended in fresh PBS in a 50ml Falcon tube, then centrifuged at 200×*g* for ten minutes at 18-20°C with the brake on. The supernatant was removed, and the lymphocyte pellet was resuspended in 10 ml of PBS. Cells were counted using a Coulter particle counter (Beckman Coulter) and re-centrifuged at 200×*g*. Supernatant was removed and the cell pellet was resuspended in a small volume of AIM-V lymphocyte medium (Invitrogen) and transferred to an appropriately sized tissue culture flask. The final lymphocyte culture was maintained at a cell density of approximately 0.5×10^6 cells/ml in a humidified atmosphere at 37°C and 5% CO_2. To simulate proliferation, lymphocyte cultures were treated with interleukin 2 (IL-2) and phytohemagglutinin (PHA) at a final concentration of 10 ng/ml and 1:100 respectively. Cells were counted every three days and media containing stimulating agents were added to maintain the cell density at 0.5×10^6 cells/ml.

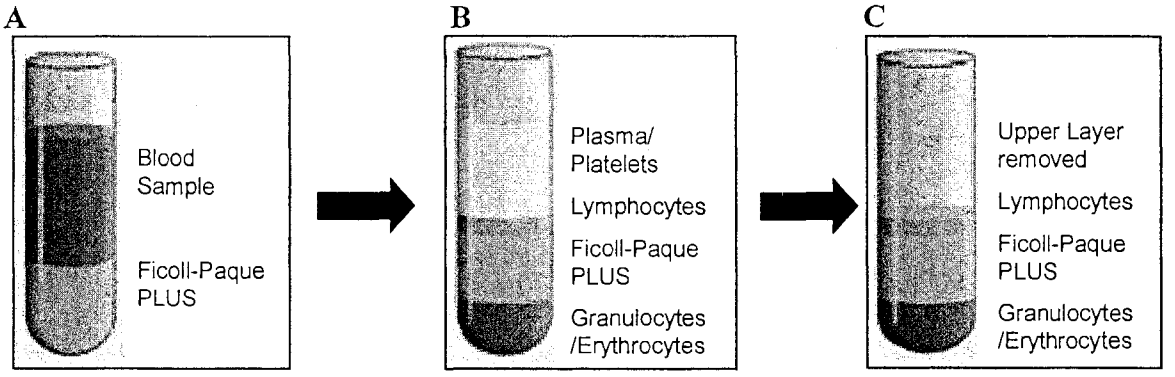


Figure 6: Diagram of the lymphocyte isolation. **A)** The blood is layered carefully over Ficoll=Plaque PLUS. **B)** After centrifugation, the blood sample is separated into respective layers. **C)** The plasma/platelet layer is removed and the lymphocytes are collected into a 50 ml Falcon tube containing PBS (Amersham product information).

3.2.3 T-Cell culture analysis

To determine the percentage of T-lymphocytes (T-cells), the lymphocyte population was stained with a CD3 antibody. This antibody detects a specific marker (CD3) located on the

surface of T-cells and lymphocyte cultures were analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences).

Analysis of non-proliferating cells was done every day for six days after isolation. Approximately 1 ml of cells (5×10^5 cells) was centrifuged at $200\times g$ for 10 minutes. The supernatant was aspirated and the cell pellet was resuspended in 100 µL PBS. When using the Beckman Coulter CD3-PE antibody, 20 µL of antibody was added to the cell suspension and incubated in the dark for 20 minutes at room temperature. When staining with the Becton Dickinson CD3-FITC antibody, 10 µL of antibody was added to the cell suspension and incubated for 30 minutes, on ice, in the dark. Analysis of stimulated lymphocytes was only done on day 3, 6 and 9 after initial lymphocyte isolation to allow the cells to aggregate and proliferate. Non-proliferating lymphocyte cultures do not have the tendency to aggregate and were not disturbed by the daily samplings.

3.2.4 BRCA1 status of participants

There were five participants in this study (Table 1). Individual 1 was a healthy control with no personal or family history of breast cancer and is unrelated to the other individuals in the study. Individuals 2, 3, 4 and 5 were sisters, whose mother was diagnosed with early on-set breast cancer at age 35 and passed away from breast cancer the at age of 38 (Figure 7). Individuals 2 and 3, did not have the BRCA1 mutation and have not had breast cancer. Individuals 4 and 5 had breast cancer and were identified to have a missense BRCA1 mutation affecting the third nucleotide on intron 17 (c.5074+3A>G) (Appendix 5 and 6).

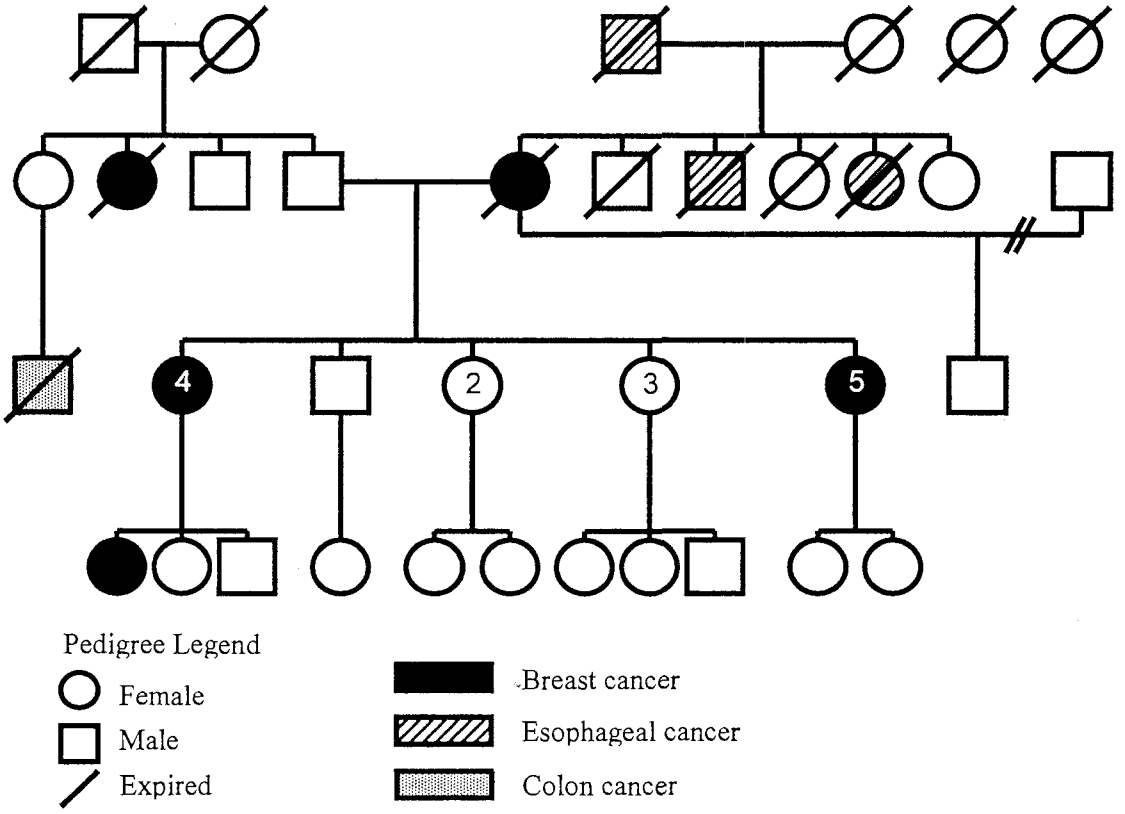


Figure 7: The family pedigree of Individuals 2-5 in this study. Individual 1 was unrelated, and therefore not included in the pedigree

This specific BRCA1 mutation is rare and has only been reported once previously in the breast cancer genetics databases. In this report coming from the Netherlands, this mutation was found to be clinically relevant. This mutation disturbs the proper splicing of the *BRCA1* pre-mRNA, which leads to the skipping of exon 17 (A.H. van der Hout 2007).

Table 1: Summary of the participants. Individual 1 was a healthy control, unrelated to the other participants in the study.

Individual	Age	BRCA1 status	Breast Cancer History	Previous treatment regimen
1	38	No mutation	None	N/A
2	47	No mutation	None	N/A
3	45	No mutation	None	N/A
4	50	Mutation	Yes	Radiation and chemotherapy
5	44	Mutation	Yes	Chemotherapy

3.3: Sulforaphane Dose Determination

Sulforaphane (SFN) was purchased from LKT Laboratories, MN. The SFN was diluted in DMSO to make a 100mM stock solution. Appropriate concentrations of SFN were added to the cells 24 hours prior to any analysis or radiation. For analyses that were completed 24 hours post radiation, cells were in the presence of SFN for a total of 48hrs. It was important to determine the safest dose of SFN that could be used without cytotoxicity. Three different assays were used to determine the cytotoxicity of SFN: the MTT assay; the alamarBlue reduction assay; and SYTOX Green staining. The assay used was dependent on whether the cell culture was stimulated to proliferate

3.3.1 Standardization of the MTT assay

The MTT cytotoxicity assay is a standard colorimetric assay that measures mitochondrial activity as an indirect indication of cell proliferation and is commonly used to determine the toxic potential of various agents. The tetrazole(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to purple formazan crystals by the mitochondrial dehydrogenases enzymes, and these crystals are solubilized in DMSO and analyzed for absorbance at 500-700 nm (Mosmann 1983). This assay is most sensitive when the cells are in log growth phase and optimal plating densities and incubation lengths were determined prior to the cytotoxicity assays. Cells were seeded in a 96-well plate at varying densities of MCF-7 cells (0-3.5×10^4cells/well) or stimulated lymphocytes (0-1.5×10^5cells/well) in eight wells per dilution. When using the adherent MCF-7 cell line, the cells were allowed to adhere overnight. After incubation for 24 or 48 hours, 15 µl MTT (5mg/ml) was added to a final concentration of 0.5 mg/ml. The plate was incubated for an additional four hours before the medium was removed, and 50 µL DMSO was added to dissolve the formazan crystals. The 96-well plate was placed in

the microplate reader. The absorbance values were read on a microplate reader (Powerwave xs, Biotec) at two wavelengths, 570 nm and 650 nm. The growth curve generated was used to determine optimal seeding densities that would produce an absorbance between 0.3 and 1.2 absorbance units. This density was used for subsequent cytotoxicity assays.

3.3.2 The MTT *in vitro* assay

The stimulated lymphocytes were seeded in a 96-well plate at the optimal concentration per well, as determined from the standard growth curve and cells were treated with increasing concentrations of SFN. Since SFN is dissolved in DMSO, which could be cytotoxic to cells at high concentrations, controls were treated with the highest dose of DMSO used in the experiment. All cells were maintained accordingly and absorbance values were determined as previously discussed. Wells containing medium and MTT only were used as a blank and subtracted from absorbance values. Absorbance values for treated cells were reported as a percentage of the untreated cells.

3.3.3 Standardization of the alamarBlue reduction assay

Similar to the MTT assay, the alamarBlue reduction assay is an indirect assay of cell viability. The alamarBlue assay is based on a colourimetric change that occurs in the alamarBlue reagent as it undergoes reduction to a pink colour from its oxidized blue form. This reduction of the alamarBlue reagent occurs when cells are proliferating and are therefore creating an excess of electrons in the growth medium which induce the colour change from blue to pink. The colourimetric change in the alamarBlue reagent can be analyzed either spectrophotometrically, or by fluorescence emission of the reduced alamarBlue (Bopp and Lettieri 2008). This assay is most sensitive when the cells are in log growth phase, and as with the MTT assay, optimal plating densities and incubation lengths were determined prior to the cytotoxicity assays. Cells

were seeded in a 96-well plate in 100 µl media/well using a range of stimulated lymphocytes (0-3.5×10^4cells/well), in eight replicate wells per dilution. The alamarBlue reagent was added five hours prior to analysis at a 1:10 dilution, at 19 hours and 43 hours for the 24 hour and 48 hour analysis time points respectively. Using a fluorescence microplate reader (FL_X 800), the plate was excited at 530-560 nm and the fluorescence was read at 600 nm.

3.3.4 The alamarBlue reduction *in vitro* assay

This was carried out in a similar fashion as the MTT *in vitro* assay. Cells were plated and treated with serial dilutions of SFN. Untreated cells were used as a control. All cells were maintained accordingly and fluorescence values were determined as previously described. Wells containing medium and alamarBlue only were used as a blank and subtracted from fluorescence values. Fluorescence values for treated cells were reported as a percentage of the untreated cells.

3.3.5 The SYTOX Green viability assay

SYTOX Green is a dye used to differentiate between damaged and healthy cells. Live, healthy cells have an intact membrane that is impermeable to the dye. Dead cells however, have damaged cell membranes, and allow the dye to enter the cell and bind to the DNA in the nucleus. One milliliter of cell culture was centrifuged for seven minutes at $100\times g$. The media was aspirated and cells were incubated with 10 nM SYTOX green in PBS for 30 minutes at 37°C. The excitation and emission wavelengths for SYTOX Green dye were 408 nm and 523 nm respectively, and analyzed by flow cytometry using the FL-1 detector of the FACSCalibur flow cytometer (BD Biosciences). A dose range between 0-20 µM of SFN was administered to non-proliferating lymphocytes 24 hours prior to analysis.

3.4 Irradiation Procedures

Experiments completed between September 2006 and April 2, 2008 used a Cobalt-60 Unit as the source of γ-radiation. Experiments done after April 2, 2008 used a linear accelerator emitting 6 MV X-rays as the radiation source, as the Cobalt unit was no longer available for research use. Both of these units were located in the Radiation Therapy department at Thunder Bay Regional Health Sciences Centre- Regional Cancer Care and irradiation was done by a medical physicist. The irradiation procedures were optimized to determine that each cell received a uniform dose of radiation. Different experimental setups were required for the irradiation of adherent and suspension cell cultures.

3.4.1 Adherent cells

To prepare for the irradiation of adherent cell cultures, the media was aspirated and the cells were washed twice with PBS. A layer of PBS at a depth of 5-7 mm was left on the cells to absorb the γ-radiation. The plate was placed on a solid water phantom to ensure adequate buildup and backscatter of the radiation and the radiation field was set at 15 cm×15 cm. The radiation source was at a distance of 100 cm above the cell monolayer.

3.4.2 Suspension cells

To prepare for the irradiation of suspension cell cultures, the cells were shaken and thoroughly pipetted in order to dispense any clumps of cells. A 5 ml vial was completely filled with cells, and the lid was sealed with Parafilm (Figure 8A). The vial was placed inside a weighted plastic sleeve and suspended in a tank filled with water (Figure 8B). The gantry of the radiation source was rotated to 90° and half the radiation dose was given from this angle, followed by a second half-dose delivered at 270° (Figure 8C).

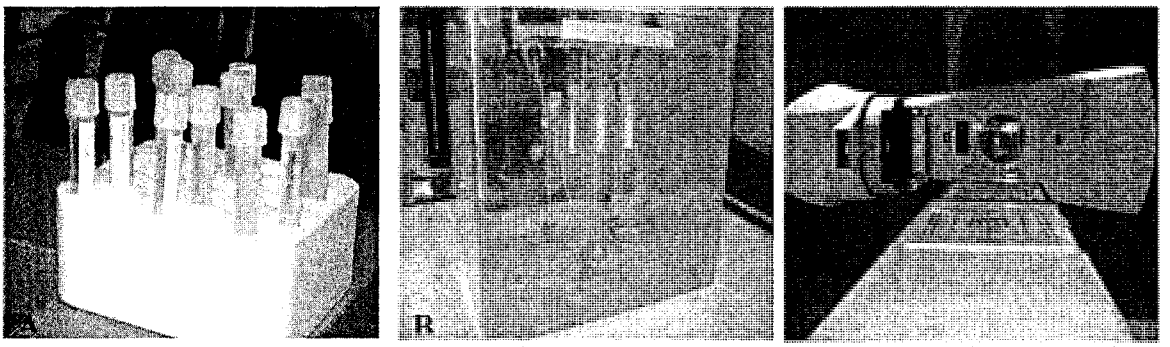


Figure 8: Irradiation set up for suspension cultures. **A)** A 5 ml vial sealed with Parafilm. **B)** Vials in the contained in the plastic sleeves suspended in the tank of water. **C)** The delivery of radiation.

3.5 Determination of irradiation dose

A patient undergoing radiation therapy is typically given a daily dose of 200 cGy. To determine if this dose was sufficient to cause detectable damage in the cell lines and lymphocyte cultures, a radiation dose response curve was generated. Cells were irradiated at a range of doses (0, 25, 50, 100, 200, 400, 600, 800 and 1000 cGy), and cell viability was assessed using the MTT assay, alamarBlue assay, and SYTOX Green analysis. The same protocols were followed as previously outlined (see sections 3.3.2, 3.3.4 and 3.3.5). Experiments were repeated three times.

3.6 Cellular antioxidant analysis

3.6.1 Glutathione redox ratio

Concentration of total glutathione (GSSG+GSH) was measured in cells by the glutathione reductase/5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) recycling assay as described previously in (Anderson 1985) with some modifications. The rate of DTNB formation was followed at 412nm and is proportional to the sum of GSH and GSSG present. Cells at the appropriate density were homogenized in 5% sulfosalicylic acid (SSA) and the cell homogenate was centrifuged for 10 minutes at 10,000×*g*. Solubilized GSSG alone was measured by treating the SSA supernatant with 2-vinylpyridine and triethanolamine. After 60 minutes, the derivitized samples were assayed as described above in the DTNB-GSSG reductase recycling assay. GSH

values were calculated as the difference between total (GSSG+GSH) and GSSG concentrations. Based on the GSH values, the redox ratio (GSH/GSSG) was determined as an indicator of oxidative stress.

3.6.2 The alamarBlue reduction assay

The alamarBlue reduction assay can be used to determine the antioxidant potential of the cell, and is an indirect measure of cell proliferation. The level of fluorescence indicates the status of the cellular reducing environment. The same protocol was followed as above (3.3.4).

3.7 DNA damage detection

3.7.1 The Comet Assay

The comet assay (single cell gel electrophoresis) is used to detect the amount of DNA damage in a single cell (Sanchez-Suarez et al. 2008). In preparation, for this assay, cell lysis solution (Trevigen) was chilled at 4°C for at least 20 minutes prior to use. Low melting point agarose (Trevigen) was melted in a boiling water bath for five minutes and divided into 200 µl aliquots in microcentrifuge tubes and allowed to cool in a 37°C water bath for at least 30 minutes prior to the addition of cells. During this time, the Comet slides (Trevigen) were warmed in a 37 °C incubator. Cells were suspended in PBS at a concentration of 1×10^5 cells/ml and were combined with the low melting point agarose at a ratio of 1:10 (cells to agarose), and then 75 µl of the sample was spotted onto two circular areas of the comet slide. The slides were refrigerated at 4°C for approximately ten minutes to allow the agarose to set. The slides were then immersed in the pre-chilled lysis solution overnight. Following lysis, the slides were removed from lysis buffer and submerged in 50 ml of freshly prepared alkaline solution, pH>13 (300 mM NaOH , 1 mM EDTA) in the dark, for one hour. The slides were placed in a horizontal electrophoresis apparatus, equidistant from the electrodes. The alkaline solution was carefully

poured so that the slides were just covered and alkaline electrophoresis was carried out at 4°C. A voltage of one volt per centimeter (length of the electrophoresis apparatus) was applied for 30 minutes. The comet slides were then rinsed three times in ddH_2O for five minutes each, immersed in ethanol for five minutes and left to air dry overnight. On the day of analysis, 50 µl of diluted SYBR Green (1 µl of 10000×SYBR Green I Stain in 10 ml of TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA)) was placed onto the sample. The excitation wavelength for SYBR Green is 497 mn, and the fluorescence emission of SYBR Green bound to DNA is 520 nm. Cells were scored by fluorescence microscopy using the scoring legend below (Figure 9). This scoring system was based on the amount of visible DNA damage. A score of 1 was the lowest when all DNA was still contained in a "head" of the comet, and a score of 4 was the highest, where there was a hedgehog effect with the majority of DNA seen in the tail (adapted from Collins 2004; Jaloszynski et al. 1997). One hundred cells were scored for each slide.

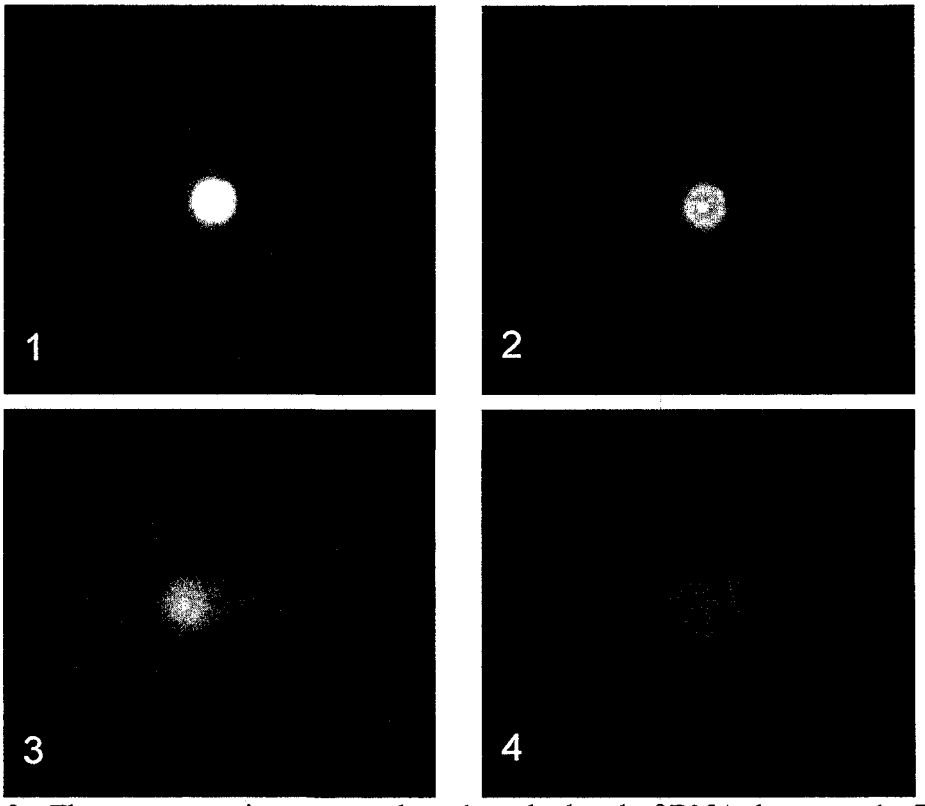


Figure 9: The comet scoring system, based on the level of DNA damage. 1= The DNA was contained in the comet "head". 2= DNA escaped the head, creating a "halo" effect. 3= The majority was still in the head, but a there was a definite "tail". 4= The "hedgehog" effect, where the majority of DNA was in a large, diffuse tail.

After scoring each slide, the number in each category was counted and an average degree of DNA damage (DD) was calculated with the following formula:

$DD = (n_2 + 2n_3 + 3n_4)/100$, where n_2 - n_4 = number of comets in categories 2-4.

3.7.2 Flow cytometric analysis of γ-H2A.X

The histone protein, H2A.X becomes phosphorylated at serine 139 in response to DNA double strand break (γ-H2A.X), and maximum phosphorylation is achieved one hour post damage induction (Olive 2004). Phosphorylated H2A.X is referred to as γ-H2A.X. After treatment and incubation, 1.0×10^6 cells in a single cell suspension were centrifuged at $200\times g$ for ten minutes and resuspended in 300 µl of PBS and fixed with 700 µL of 99.9% ethanol that was added while vortexing. The ethanol-fixed samples were stored at -20°C and remained stable

until the time of analysis. On the day of analysis, 1 ml of cold TBS (Tris buffered saline) was added to the cells and centrifuged at 800×*g*. The cell pellet was resuspended in 1ml of cold TST (1×Tris buffered saline containing 4% fetal bovine serum, 0.1% triton X-100) for ten minutes to permeabilize and rehydrate the cells. Samples were centrifuged and resuspended in 200μL TST containing mouse monoclonal γ-H2A.X antibody (Upstate Catalog # 05-636) diluted in 1:500 TST. Covered samples were shaken on a shaker platform at room temperature for two hours. After the incubation, samples were centrifuged and washed three times in TST for five minutes each time. The samples were then resuspended in 200 μL TST containing secondary antibody (Cy-3 anti-mouse IgG, Jackson catalog # 115-165-003) diluted 1:200 in TST. Covered samples were shaken on a shaker platform at room temperature for one hour. Cells were pelleted and resuspended in TBS with 4% fetal bovine serum and analyzed for fluorescence by flow cytometry. Cy 3 is excited at 550 nm and emits at 570 nm, which is detectable in the FL-2 detector (585/42 nm). Unstained samples and samples stained with only primary antibody or with only the secondary antibody were used to determine the appropriate cytometer instrument settings, and to determine background non-specific antibody fluorescence.

3.8 Statistical Analysis

Optimization experiments were completed on three separate occasions unless otherwise stated. The mean was calculated and error bars represent the standard deviation of the mean. When using the patient samples, the experiments were performed in duplicate. The mean was calculated and the error bars represent the standard deviation from the mean. An in depth statistical analysis was not done because of the limited sample size.

4. Results

4.1 Effect of ionizing radiation on the redox status of MCF-7 breast cancer cell line

To investigate the impact of ionizing radiation and SFN on a breast cancer cell line without any known BRCA1 mutation, initial experiments were done using MCF-7 cells.

4.1.1 Determining the cytotoxicity of SFN

A standard curve was done to determine the optimal plating density of MCF-7 cells for the MTT cytotoxicity assay (Figure 10A). The optimal plating density was chosen by selecting a cell count that produced an absorbance between 0.7 and 1.2. From these results, a plating density of 2.5×10^4 cells/well was chosen, giving an absorbance reading of approximately 0.8 (Figure 10A). MCF-7 cells were treated with increasing concentrations of SFN for 24 hours to determine its cytotoxicity (Figure 10B). A dose of 40 μM was found to be cytotoxic to 50% of the cell population (LD_{50}), and 20 μM yielded 80% viability (Figure 10B).

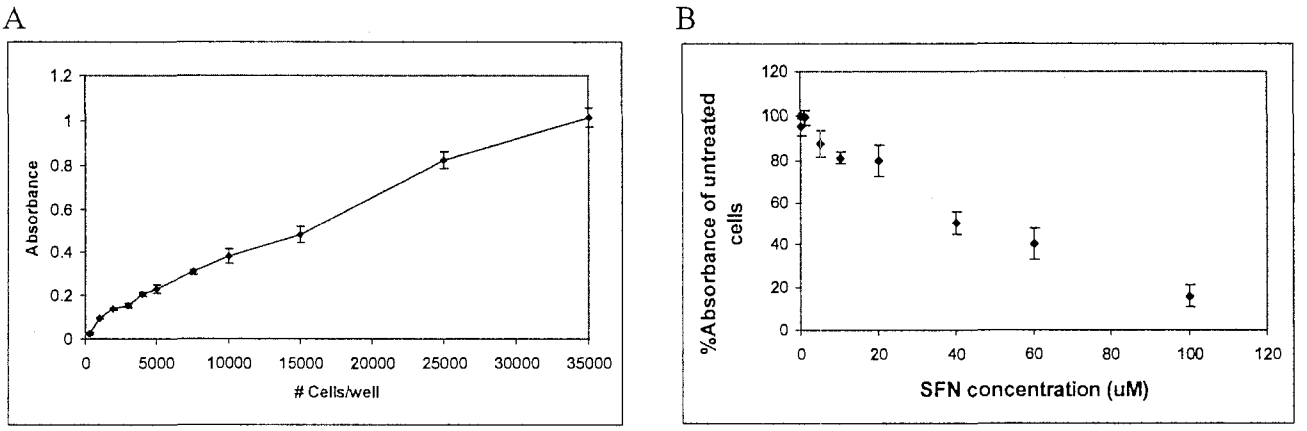


Figure 10: The MTT assay. **A)** The standard curve for MCF-7 cancer cells to optimize plating densities for a 24hr end point, which was determined to be ~2.5×10^4 cells/well. **B)** SFN cytotoxicity as measured by the MTT assay. Error bars represent standard deviation.

4.1.2 Glutathione analysis

To determine the effect of SFN on cellular redox potential of SFN, MCF-7 cells were pre-treated for 24 hours with a concentration range of 0-20 μM. Cells were harvested and resuspended at a density of 2.0×10^5 cells/ml. Total GSH, reduced GSH, and GSSG were

measured using a standard colourimetric technique and the redox ratio (GSH/GSSG) was determined (Figure11).

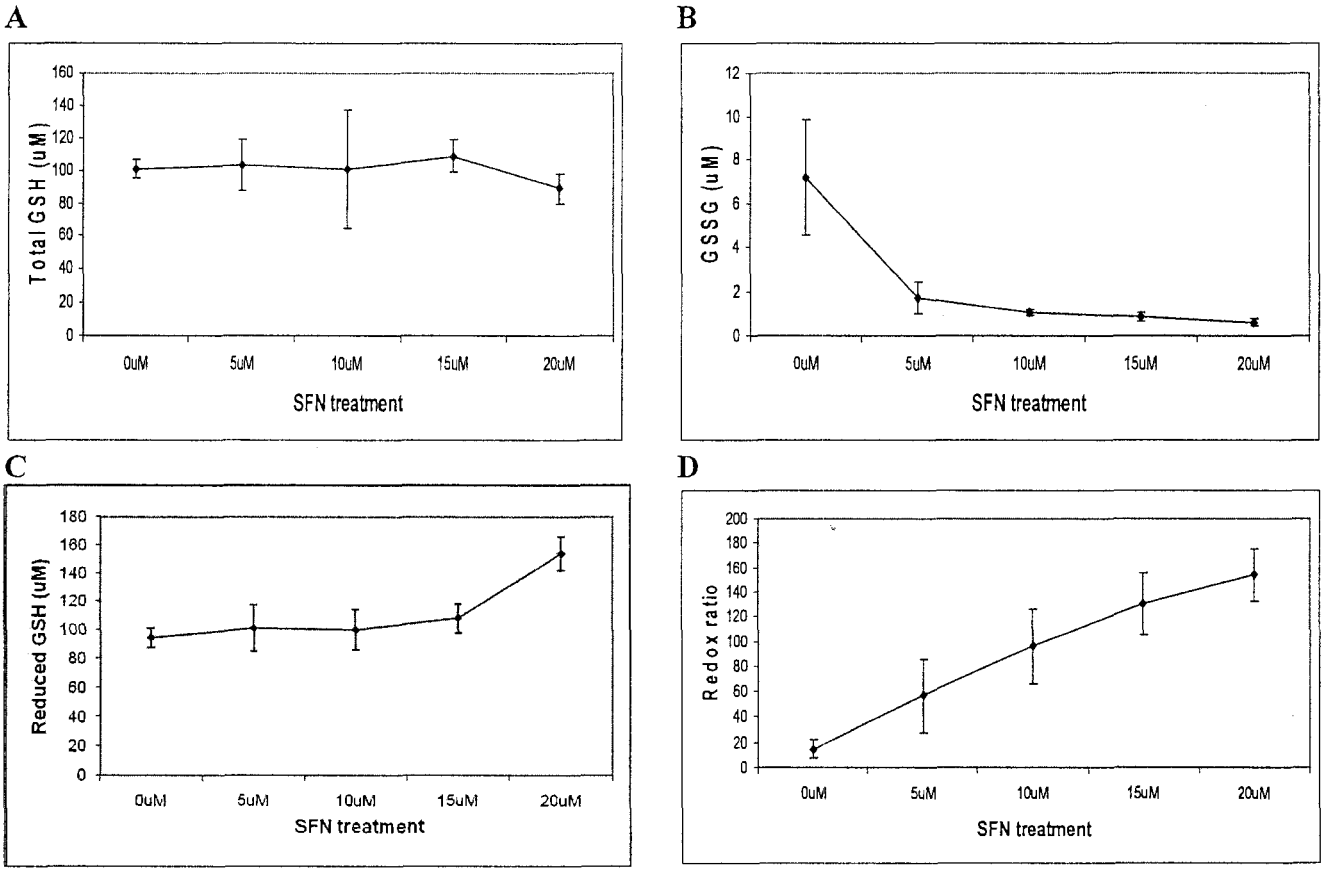


Figure 11: GSH analysis of MCF-7 cells following pre-treatment with a range of SFN doses. **A)** Total GSH. **B)** Oxidized glutathione (GSSG). **C)** Reduced GSH. D) The redox ratio. This experiment was done in triplicate and the error bars represent standard deviation from the mean.

With increasing concentration of SFN up to 20 μM, there was a notable increase in the redox ratio in MCF-7 cells, mainly due to the decrease in GSSG. This effect was evident at the highest dose of 20 μM. The results showed the antioxidant potential of SFN, and 20 μM was used for subsequent studies of the antioxidant on the effects of ionizing radiation. Based on this finding, MCF-7 cells were pretreated with 20 μM SFN 24hr prior to ionizing radiation exposure.

4.1.3 Effects of SFN in MCF-7 cells following ionizing radiation

MCF-7 cells were treated with a dose of 20 μM SFN for 24 hours prior to exposure of cells to 0, 200 or 400 cGy of ionizing radiation. One hour post irradiation, cells were harvested and resuspended at 2.0×10^5 cells/ml. GSH analysis was done and the redox ratio was calculated (Figure 12).

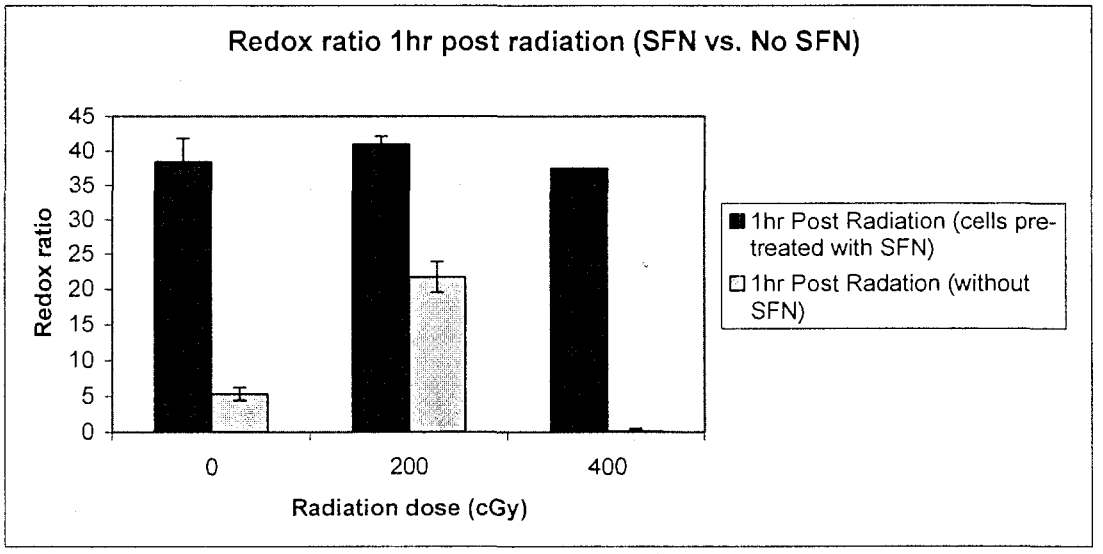


Figure 12: Redox ratio of MCF-7 cells treated with SFN 24 hours prior to irradiation exposure. The redox ratio was calculated one hour after post radiation treatment. Error bars represent the standard deviation from the mean.

GSH analyses (Figure 12) show that cells given SFN had a better redox ratio than those not given SFN after ionizing radiation exposure.

4.1.4 Summary of results obtained using the MCF-7 cell line

Using the MTT assay, it was determined that a dose of SFN up to 20 μM was minimally cytotoxic in MCF-7 cells and increased the GSH redox ratio by decreasing the intracellular concentration of GSSG (Figure11). When MCF-7 cells were pretreated with 20 μM of SFN 24 hours prior to ionizing radiation, the redox ratio remained high compared to cells that were not

pretreated with the antioxidant (Figure 12). The results showed that SFN was an effective antioxidant in cells that were exposed to ionizing radiation.

4.2 Non-proliferating lymphocytes

The focus of this study was to measure the radiation response in lymphocytes obtained from individuals with BRCA1 mutations. Primary lymphocytes were chosen as a model because they are a good representation of a normal, non-cancerous cell in a patient's body when exposed to ionizing radiation and cytotoxic agents. In our laboratory, primary lymphocytes have been observed to have very different responses to ionizing radiation and cytotoxic agents than lymphoblastoid cell lines with BRCA1 mutations and other cell lines derived from tumor tissue (unpublished data).

4.2.1 Radiation response of non-proliferating lymphocytes versus proliferating lymphocyte cultures

Previous studies have shown that stimulating agents such as PHA and IL-2 confer some radiation resistance to primary lymphocytes (Lavin and Kidson, 1977). Enzymes needed for DNA replication are also required for DNA repair. By stimulating the lymphocyte culture, these enzymes are readily available to repair the damage caused by radiation exposure. Lymphocytes extracted from the same blood sample were divided into two cultures, proliferating with PHA and IL-2, and non-proliferating. These cultures were exposed to ionizing radiation and analyzed for DNA double stranded breaks using a conjugated fluorescent antibody that detects the phosphorylated histone protein, γ-H2A.X (Figure 13). Upon exposure to DNA damaging agents, such as ionizing radiation, DNA double strand breaks are induced and H2A.X becomes phosphorylated at Ser 139, initiating the DNA repair process (Figure 13). These cells were

exposed to 200 cGy ionizing radiation, and allowed to incubate for one hour, before fixing in ethanol.

A B

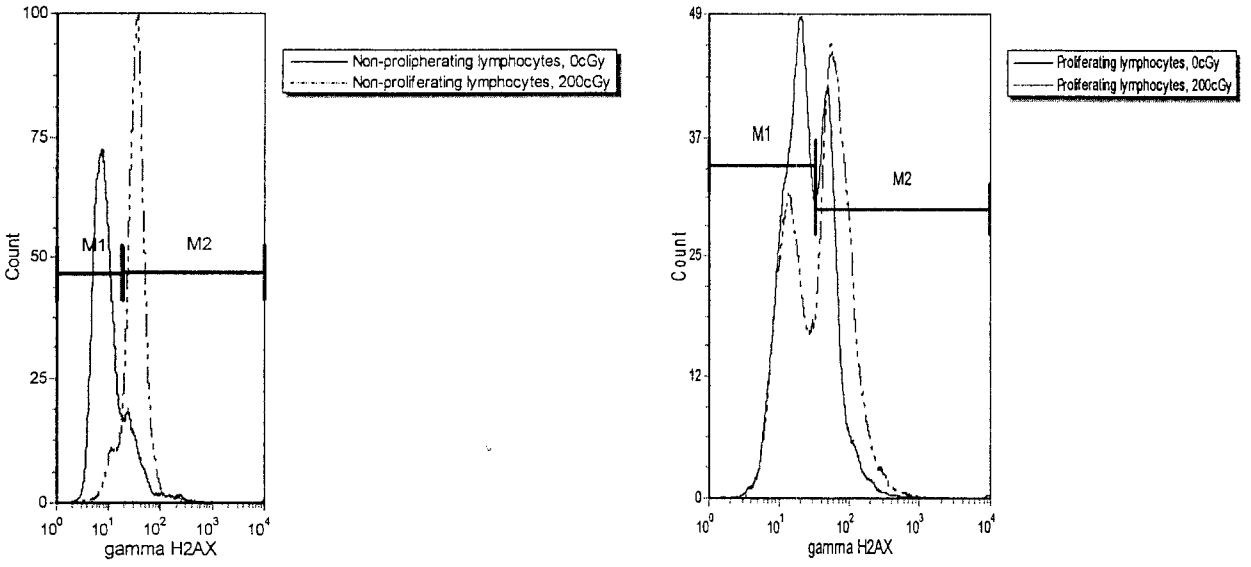


Figure 13: Comparison of radiation response in **A)** non-proliferating and **B)** proliferating lymphocytes using the γ-H2A.X conjugated fluorescence antibody and analyzed by flow cytometry. In each sample 1.0×10^4 cells were analyzed (n=3).

In the treated, non-proliferating sample, there is a 70% increase in γ-H2A.X positive cells (M2) above the background level seen in the untreated control (M1) (Figure 13A). This was striking compared to the 20% increase in γ-H2A.X positive cells above the untreated background level in the proliferating cells (Figure 13B). The high background level of γ-H2A.X positive cells was due to the presence of small γ-H2A.X foci that occur independently of DNA double strand break induction (McManus and Hendzel 2005). In proliferating cells, the level of these small γ-H2A.X foci are at the highest level during the G_2/M phase of the cell cycle (McManus and Hendzel 2005).

4.2.2 Determination of T-cell population

The T-lymphocyte (T-cell) population was determined using a T-cell specific CD3 stain and analysis using the flow cytometer (Figure 14). A cell population appeared consistently in the same spot on a forward scatter (FSC) vs. side scatter (SSC) dot plot (Figure 14A). This population was identified as the population of interest and a gate was applied. When the gated population was analyzed and plotted on a FSC vs. FL-1 dot plot, the population appeared in the first decade of fluorescence (close to the y-axis) in an unstained sample (Figure 14B). After staining the cells with a CD3-FITC antibody, this same population stains positively for the CD3 marker and the mean fluorescence intensity increased (15C) therefore it was concluded that this population was in fact made up of T-cells. In each sample 3.0×10^4 cells were analyzed.

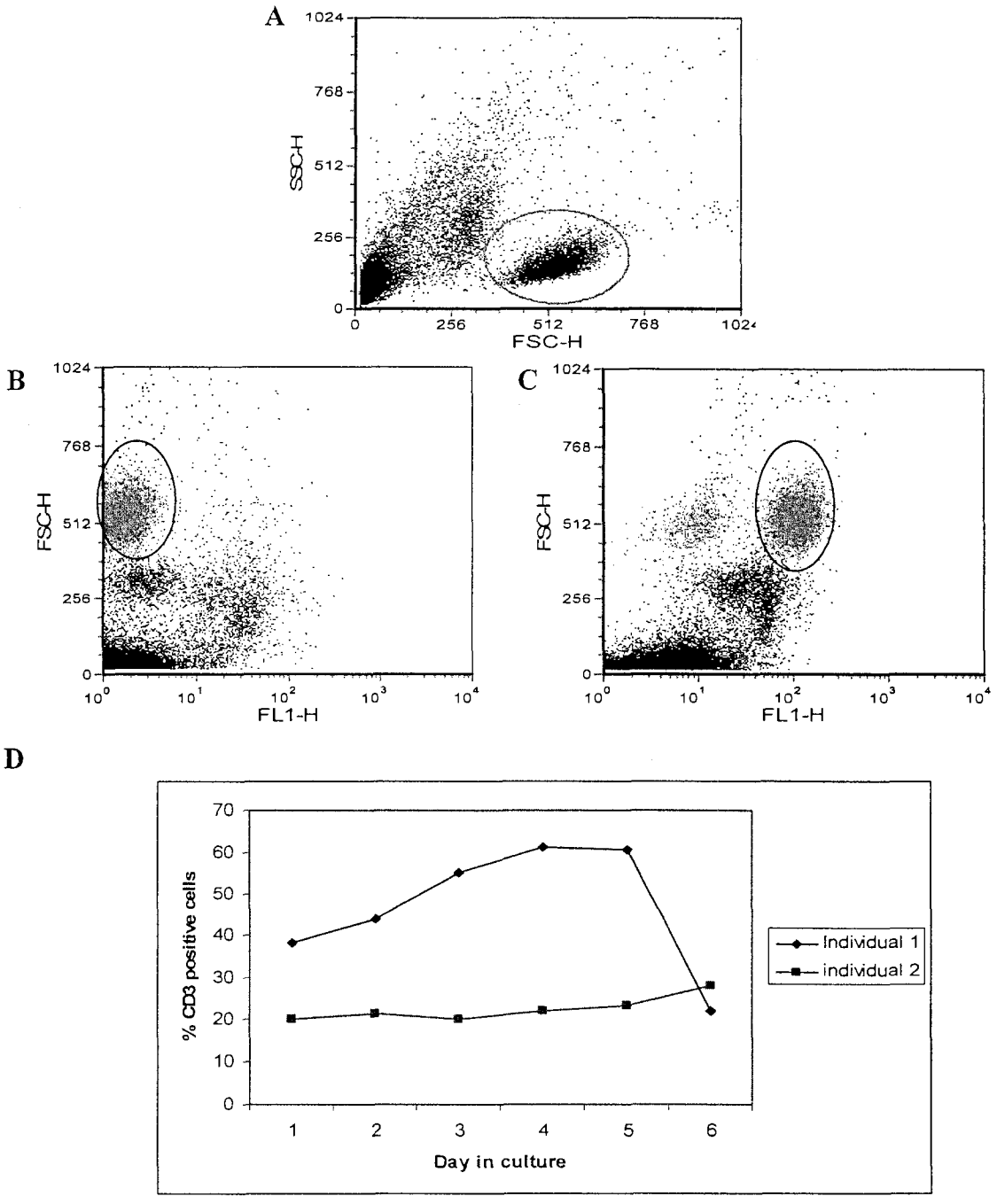


Figure 14: CD3 analysis of the T-cell population in non-proliferating lymphocytes. **A)** A cell population consistently appears in the gated region of a SSC vs. FSC dot plot. **B)** The area where this population appears in an unstained sample. **C)** This same population shifted to the right when a CD3 antibody is added, staining positively as T-cells. **D)** Length of time T-cells persist in non-proliferating tissue culture conditions.

The T-cell population only persisted for a limited amount of time when stimulating agents (such as IL-2 and PHA) were not added (Figure 14D). The number of T-cells also varied

between individuals. Based on these results, any experiments done with non-proliferating lymphocytes were done between Day 2 and Day 5 after isolation.

4.2.3 Determination of SFN pretreatment

To determine the maximal non-cytotoxic concentration of SFN that would be used to pretreat non-proliferating lymphocytes, a SYTOX Green stain was used. A range of 0-20 μM SFN was given to aliquots of cells 24 hours prior to analysis with the flow cytometer (Figure 15).

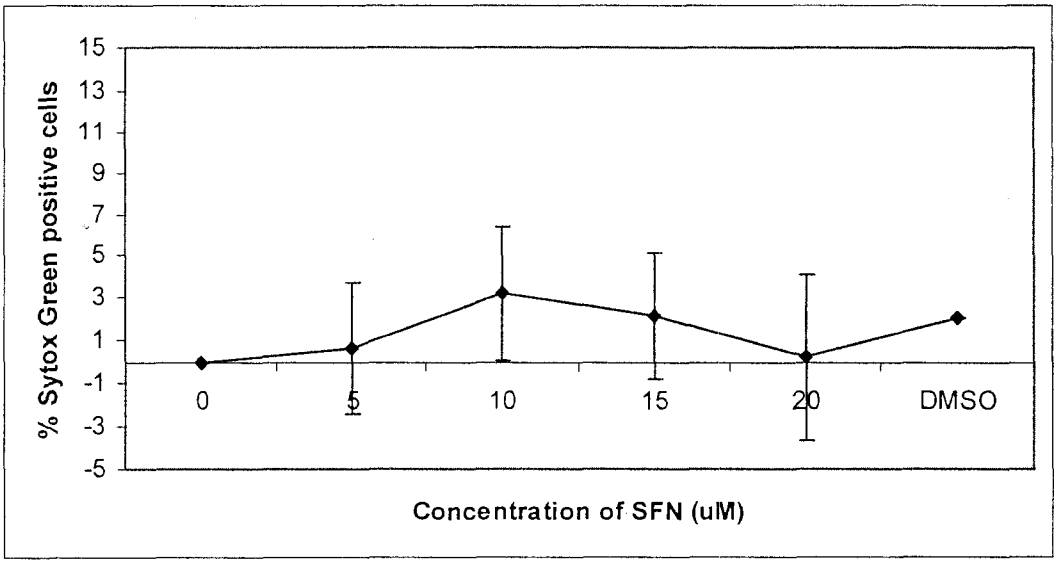


Figure 15: SFN dose response in non-proliferating lymphocytes using SYTOX Green analysis with flow cytometry. Non-proliferating lymphocytes were treated with increasing concentrations of SFN for 24hrs and 3.0×10^4 cells were analyzed (n=3). Error bars represent standard deviation from the mean.

SYTOX Green is a measure of viability based on membrane integrity. These experiments did not show an increase in membrane disruption with higher doses of SFN. Based on these experiments, a pretreatment concentration of 10 μM was chosen. According to the literature, this was at the lower end of the range of what was recommended for other cell types. A lower end concentration was chosen because primary cells are generally more sensitive to drugs and other reagents than immortalized cell lines. Non-proliferating cells do not proliferate

well in tissue culture conditions and therefore there is a very low cell count, which limited the number and type of assays that could be used for this cell type.

4.2.4 Determination of irradiation dose

To determine the sensitivity of the SYTOX Green assay to detect a decrease of cell viability after exposure to ionizing radiation, aliquots of non-proliferating lymphocytes were given increasing doses of radiation. Samples were stained with SYTOX Green and analyzed with the flow cytometer 1 hour and 24 hours after radiation exposure (Figure 16A and B).

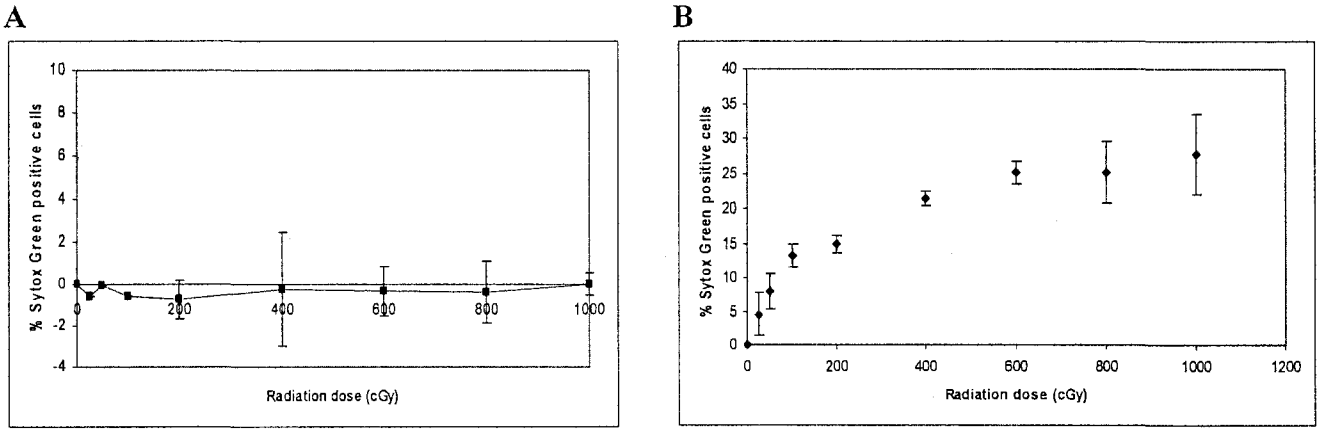


Figure 16: SYTOX Green analysis of non-proliferating lymphocytes with flow cytometry after exposure to increasing doses of radiation. Approximately, 3.0×10^4 cells were analyzed in each sample and data was normalized to account for background levels of staining. **A)** At one hour post exposure to ionizing radiation (n=2). **B)** At 24 hours post exposure to ionizing radiation (n=3). Error bars represent standard deviation from the mean.

SYTOX Green is a measure of viability that is based on membrane integrity. One hour after exposure to radiation exposure, the non-proliferating cells show very little change in membrane integrity (Figure 16A). However, at 24 hours post radiation, membrane integrity was decreased, as there was an increase in SYTOX Green positive cells at the higher doses of radiation.

4.2.5 Glutathione analysis of non-proliferating lymphocytes

Between 8×10^5 and 1×10^6 cells were needed to get a reliable glutathione reading (Figure 17).

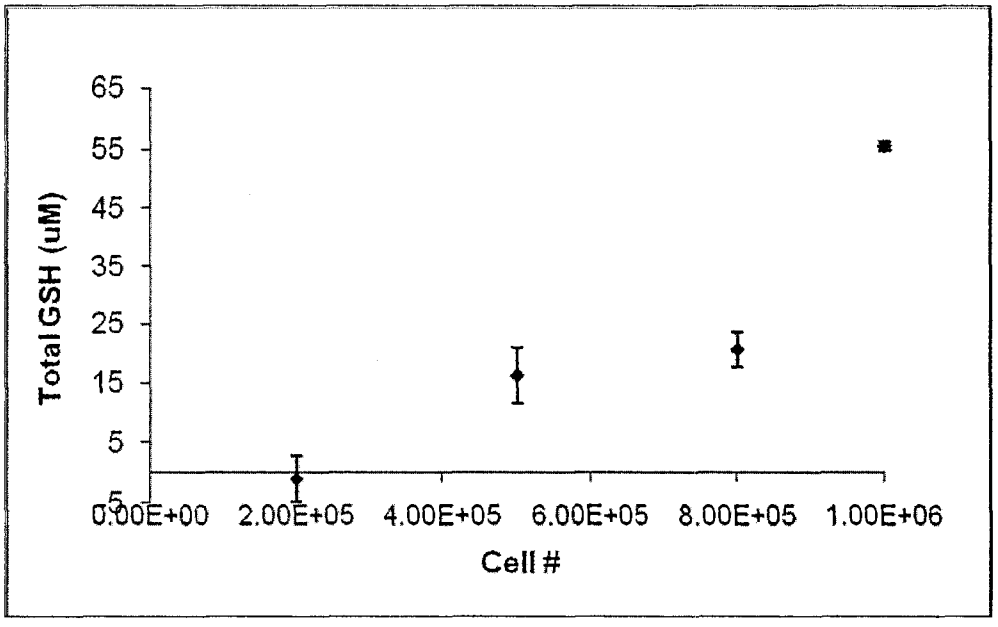


Figure 17: Total glutathione detection in increasing concentration of non-proliferating lymphocytes, starting at 2×10^5 cells/ml to 1×10^6 cells/ml (n=1, 3 replicates). Error bars represent the standard deviation from the mean.

Because such a large cell number was needed, this experiment was not pursued with non-proliferating cells. Non-proliferating cell cultures do not produce such high quantities of cells. It would not have been technically feasible to do this experiment, as well as other types of analyses using non-proliferating cells without having access to large quantities of whole blood.

4.2.6 DNA double strand break detection via the presence of γ-H2A.X.

Non-proliferating cells were treated with 10 μM SFN 24 hours prior to radiation, and fixed in ethanol one hour after radiation. Upon analysis, it appeared that SFN did protect the cells from DNA damage caused by ionizing radiation (Figure 18).

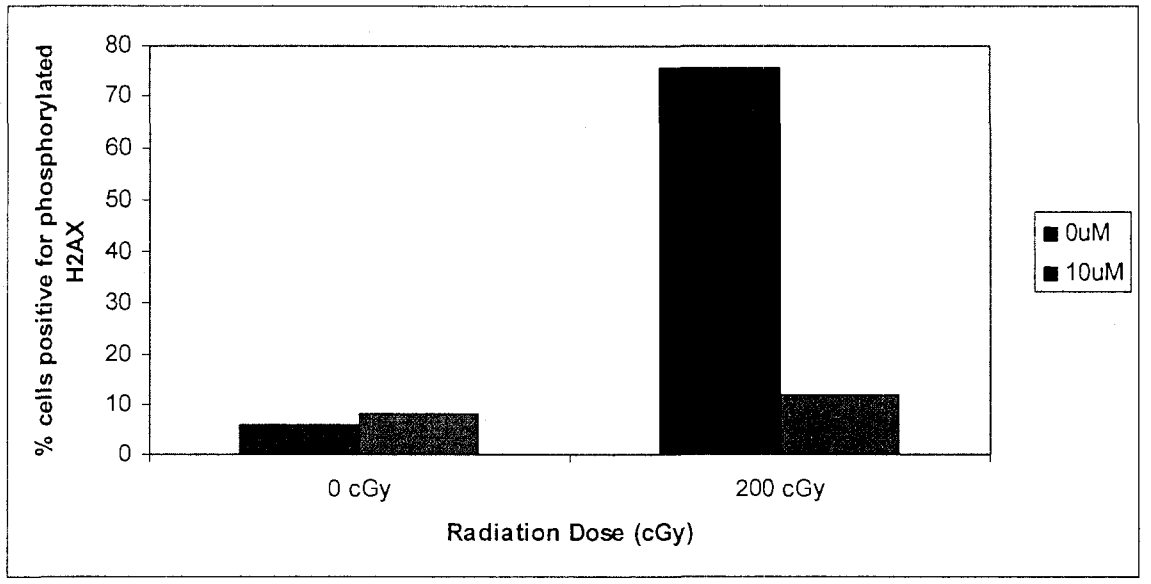


Figure 18: Flow cytometric analysis of γ-H2A.X positive cells using non-proliferating lymphocytes treated with SFN prior to irradiation exposure. Cells were allowed to recover for one hour and then were fixed in ethanol and stored at -20°C until time of analysis.

Although consistent with the finding with MCF-7 cells, the low cell numbers did not allow confidence in the results. Due to the many centrifugation steps involved in sample preparation, only approximately 5.0×10^2 cells were left by the end of the protocol.

4.2.7 Summary of results obtained using non-proliferating lymphocytes

The main reason for choosing to work with non-proliferating cells was because adding stimulatory agents such as PHA, confers radiation resistance (Lavin and Kidson, 1977). Proliferating cells are constantly undergoing replication and replication is strongly linked to repair. This is evident when comparing the level of background γ-H-2AX positive cells in an untreated, proliferating cell sample to an untreated, non-proliferating cell sample (Figure 13).

There were obvious problems when working with non-proliferating cells. It was a heterogeneous population, with only a small population that was identified positively as T-cells which existed for a short period of time (Figure 14). The cell number was low and that limited the number and types of techniques that could be used.

A radiation dose response was generated successfully, showing that increasing the radiation dose did increase cellular membrane disruption 24 hours after radiation exposure (Figure 16B).

4.3 Proliferating lymphocytes

4.3.1 Determination of T-cell population

Aliquots of proliferating cell cultures were analyzed for CD3 on the third, sixth and ninth day after lymphocyte extraction. As expected, proliferating lymphocyte cultures yielded a higher T-cell population for a longer period of time in cell culture conditions, compared to non-proliferating cultures. Also, there appeared to be less variation in the percentage of CD3 positive cells between individuals when proliferating lymphocytes were analyzed (Figure 19).

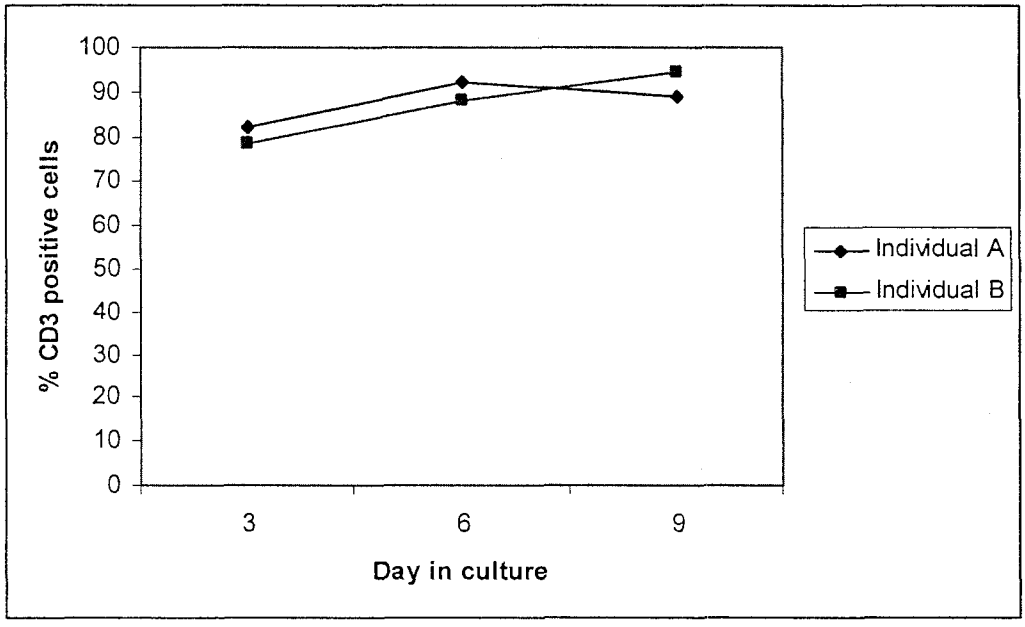


Figure 19: CD3 analysis of proliferating cell cultures using flow cytometry, 3.0×10^4 cells were analyzed per sample. (n=2).

Advantages of working with a proliferating culture were evident, including a homogeneous T-cell population, a significantly higher cell count and a longer survival time of the lymphocytes.

4.3.2 Determination of SFN pretreatment

To determine the cytotoxicity of SFN in proliferating lymphocytes, two assays were used which indirectly measure cell viability; the MTT assay and the alamarBlue reduction assay. To determine the amount of cells needed to obtain reliable fluorescence and absorbance readings, standard curves were done for both the 24 hour and 48 hour time points (Figures 20 and 21).

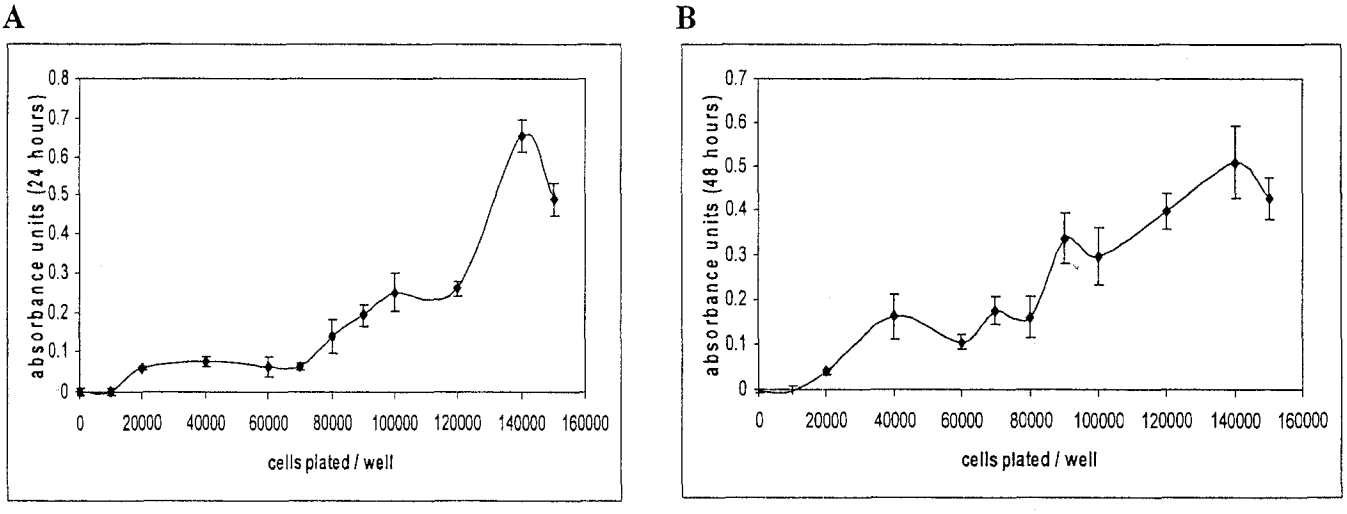


Figure 20: Standard curves for lymphocytes to optimize plating densities for both **A)** 24 and **B)** 48hr time points using the MTT assay (n=1, 8 replicates). Error bars represent the standard deviation from the mean.

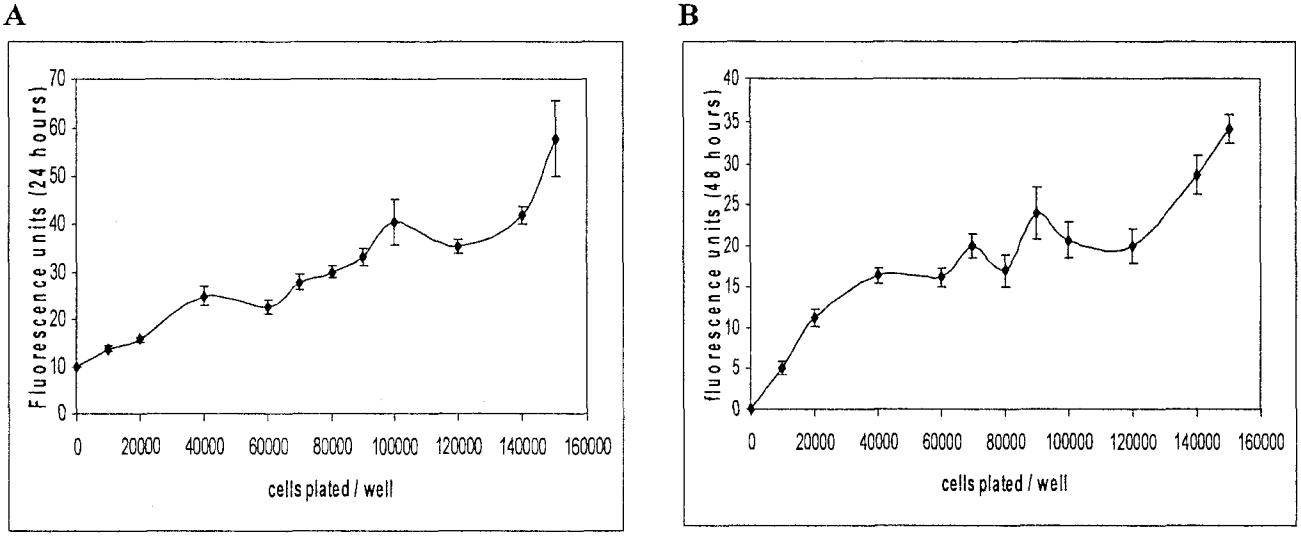


Figure 21: Standard curves for lymphocytes to optimize plating densities for both **A)** 24 and **B)** 48hr time points using the alamarBlue reduction assay. (n=1, 8 replicates). Error bars represent the standard deviation from the mean.

For both assays, the MTT assay and alamarBlue reduction assay, 100 000 cells/well (1.0×10^6 cells/ml) was found to be an appropriate initial plating density. Cell were seeded in the 96-well plate and incubated for 3 hours before increasing doses of SFN were added and the absorbance (MTT assay, Figure 22) and fluorescence (alamarBlue reduction assay, Figure 23) levels were analyzed 24 and 48 hours later.

A B

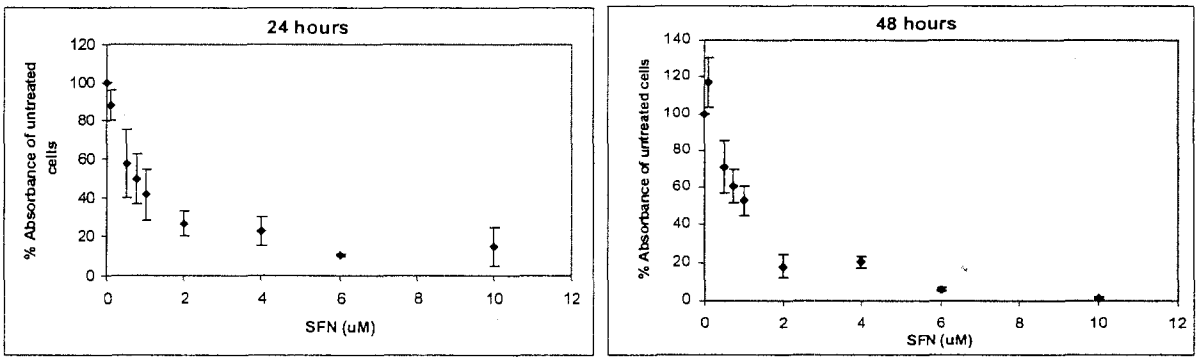


Figure 22: SFN dose response in proliferating lymphocytes using the MTT assay. **A)** Analysis at 24hours. **B)** Analysis at 48 hours (n=3, 8 replicates each experiment). Graphs are representative of one experiment and error bars represent the standard deviation from the mean.

SFN was significantly more toxic in proliferating cells compared to the cell line and non-proliferating cells. From the MTT and alamarBlue analyses shown in Figures 23 and 24, 60-80% of cells remained viable in 0.5 μM SFN and this was the concentration used for subsequent studies.

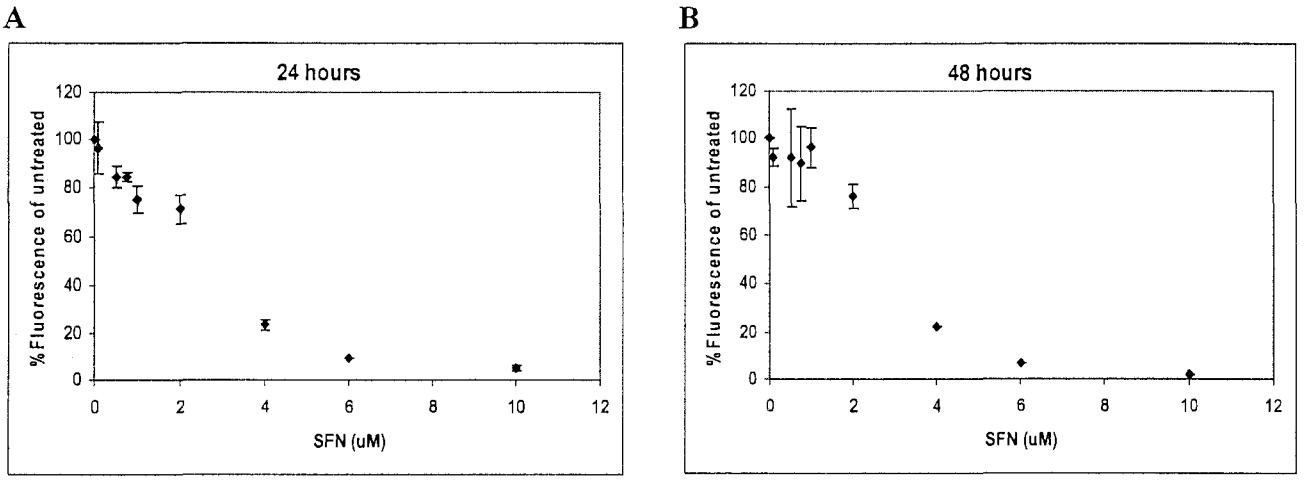


Figure 23: SFN dose response in proliferating lymphocytes using the alamarBlue assay. **A)** Analysis at 24hours. **B)** Analysis at 48 hours (n=3, 8 replicates each experiment). Graphs are representative of one experiment and error bars represent the standard deviation from the mean.

4.3.3 Determination of irradiation dose

As with the non-proliferating cell culture, SYTOX green analysis was done to assess cell viability one hour and 24 hours after ionizing radiation. Consistent with the non-proliferating cells, at one hour post irradiation, very little change was seen in membrane integrity (Figure 24).

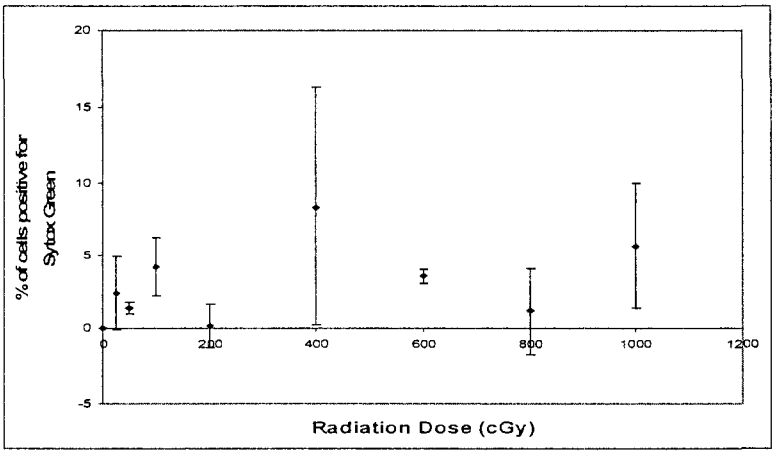
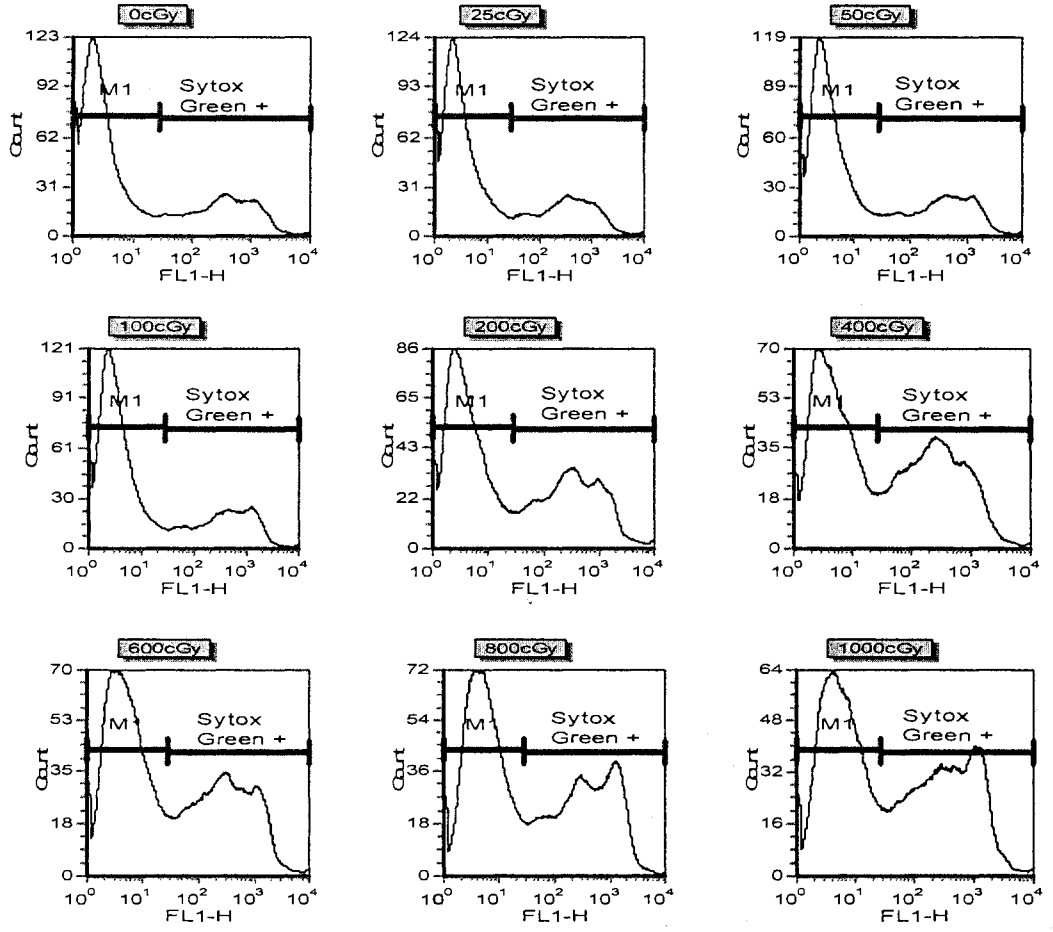


Figure 24: SYTOX Green viability analysis with flow cytometry one hour after exposure to increasing doses of ionizing radiation. Data is normalized to account for background levels. At each dose 3.0×10^4 cells were analyzed (n=3). Error bars represent the standard deviation from the mean.

However, at 24 hours after radiation, there was a definite decrease in membrane integrity as the radiation dose was increased (Figure 25). This was seen in by the increase in cell staining

with SYTOX Green in the data collection histograms (Figure 25A). There is a 15% increase in SYTOX Green positive cells at 1000 cGy (Figure 25B). At the radiation fraction used in therapy of 200 cGy, there was an 8% increase in SYTOX Green positive cells, and at 400 cGy there was a 10% increase.

A



B

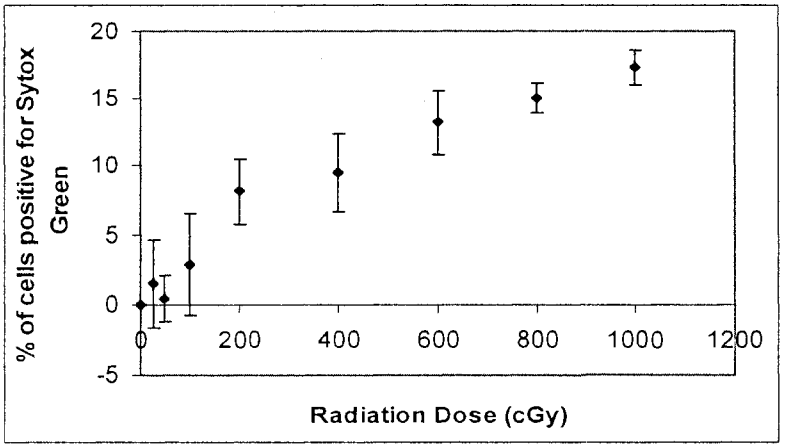


Figure 25: SYTOX Green analysis 24 hours after exposure to increasing doses of ionizing radiation. At each radiation dose, 3.0×10^4 cells were analyzed. **A)** The data collection histograms depicting the increase in SYTOX Green positive cells. **B)** The data was normalized to account for the background level of staining and plotted on a linear graph (n=3). Error bars represent the standard deviation from the mean.

Radiation dose responses were also done using the MTT and the alamarBlue reduction assay.

For both assays, cells at a concentration of 1.0×10^6 cells/ml were exposed to increasing doses of ionizing radiation and then plated in a 96-well plate (1.0×10^5 cells/well) (Figure 26 and Figure 27).

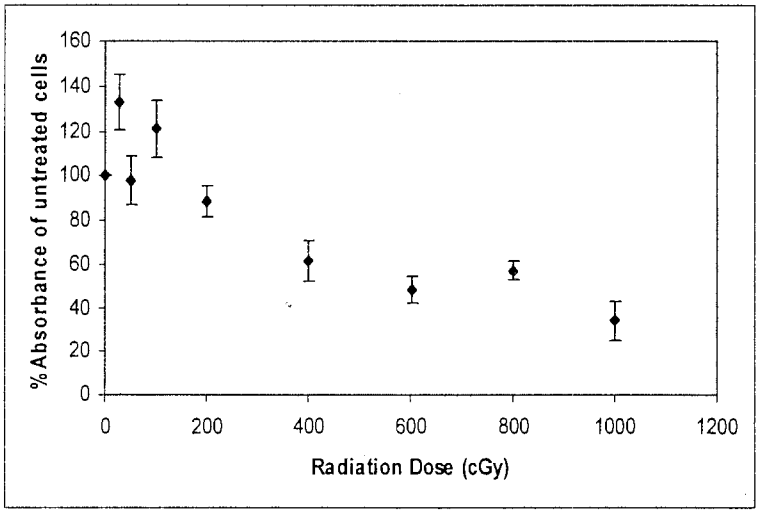


Figure 26: Radiation dose response as measured by the MTT assay, 24 hours after ionizing radiation exposure (n=3, 8 replicates in each experiment). The graph is representative of one experiment. Error bars represent standard deviation from the mean.

Consistent with the SYTOX Green viability assay, there was a dose dependent decrease in cell viability. At 200 cGy there was a 20% decrease in viability and at 400 cGy, there is a 40% decrease in viability (Figure 26).

The alamarBlue reduction assay was also consistent with the SYTOX Green viability assay in that there appeared to be a dose dependent decrease in cell viability (Figure 27). As well as being an indirect measure of cell viability, the alamarBlue reduction assay is an indirect measure of the cellular reducing environment. Comparable to results obtained using the MTT assay, at 200 cGy there is a decrease in viability, as well as in the cellular reducing environment, of 20% and 35% at 200 cGy and 400 cGy, respectively (Figure 27).

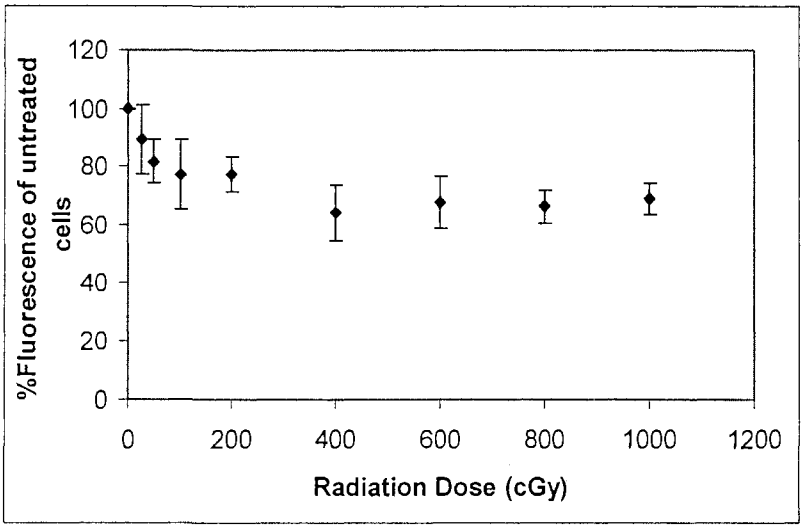


Figure 27: Radiation dose response as measured by the alamarBlue reduction assay, 24 hours after ionizing radiation exposure (n=3, 8 replicates in each experiment). The graph is representative of one experiment. Error bars represent standard deviation from the mean.

Based on these experiments, the appropriate radiation dose to achieve adequate cell damage and a decrease in the cellular reducing environment for subsequent analyses was 400 cGy.

4.3.4 DNA damage detection

Two techniques were used to detect the presence of DNA damage, analysis of γ-H2A.X and the comet assay (single cell gel electrophoresis). As previously described in section 4.2.1, γ-H2A.X is phosphorylated histone protein found at the site of DNA double strand breaks. Because cells were proliferating, there is a high level of background γ-H2A.X in the untreated control and at 200 cGy there was only a 20% increase of γ-H2A.X positive cells (Figure 28) (McManus and Hendzel 2005). At 400 cGy, there was approximately a 40% increase in cells staining positive for γ-H2A.X above the untreated control (Figure 28).

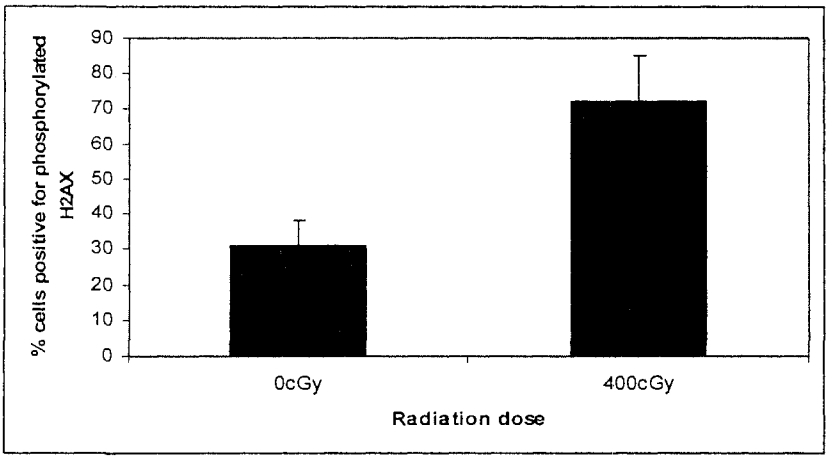


Figure 28: Flow cytometric analysis γ-H2A.X positive cells in proliferating lymphocytes that were irradiated with a dose of 400 cGy. Cells were allowed to incubate and repair for 60', and then were fixed in ethanol and stored at -20°C. A total of 1.0×10^4 cells were stained and analyzed (n=3). Error bars represent the standard deviation from the mean.

The comet assay detects the amount of DNA damage in a single cell. Cells were exposed to 0, 200 and 400 cGy and immediately resuspended at 1.0×10^5 cells/ml in PBS and mixed with the agarose gel and placed on comet slides. The scoring system based on four categories of DNA damage was used, as described previously (Figure 9), and 100 comets were scored per slide (Figure 29).

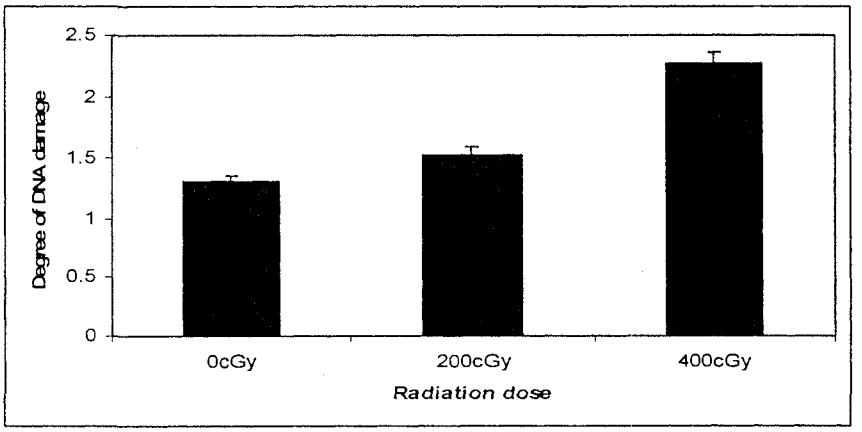


Figure 29: The comet assay using proliferating lymphocytes exposed to ionizing radiation. DNA damage at each radiation dose was determined using the four categories. One hundred comets were scored per slide (n=2). Error bars represent the standard deviation from the mean.

Cells that were given a dose of 400 cGy exhibited more damage than those that were treated with 200 cGy (Figure 30). This indicates that for both DNA damage detection assays, a

dose of 400 cGy is sufficient to induce a significant amount of DNA damage detectable by comet assay.

4.3.5 Summary of results obtained using proliferating lymphocytes

There were advantages to working with proliferating cell cultures. The first of which was that the T-cell population persisted for at least nine days after the lymphocyte isolation, and because of the addition of the stimulatory agents, there was a much larger number of cells available.

Based on the MTT assay (Figure 22) and the alamarBlue reduction assay (Figure 23), a dose of 0.5 μM SFN did not induce cytotoxicity in proliferating lymphocytes. It was decided that 0.5 μM SFN would be the dose used for the 24 hour pretreatment in subsequent experiments.

The radiation dose response was analyzed using three different experiments, the SYTOX Green viability assay (Figure 25), the MTT assay (Figure 26) and the alamarBlue reduction assay (Figure 27), which also measured the cellular reducing environment. A radiation dose of 400 cGy induces adequate cell death to produce the features of cell death that could be measured by the three assays.

The DNA detection assays, the γ-H2A.X (Figure 28) detection conjugated antibody system and the comet assay (Figure 29), also required a dose of 400 cGy to be sufficient to induce a significant amount of detectable damage.

Based on these results, it was decided that when working with patient samples, the pretreatment of SFN would be 0.5 μM and the radiation dose would be 400 cGy.

4.4 Samples from study subjects

In this section, results for each individual participant are discussed separately. In the previous section, a dose of 0.5 μM SFN was found to be tolerable when cells were treated for 24 hours. A 400 cGy dose of ionizing radiation was found to induce detectable effects in all five experimental techniques: the MTT assay, the alamarBlue reduction assay, the comet assay, γ-H.2AX flow cytometry analysis for DNA double strand breaks and the SYTOX Green viability assay (Figure 30).

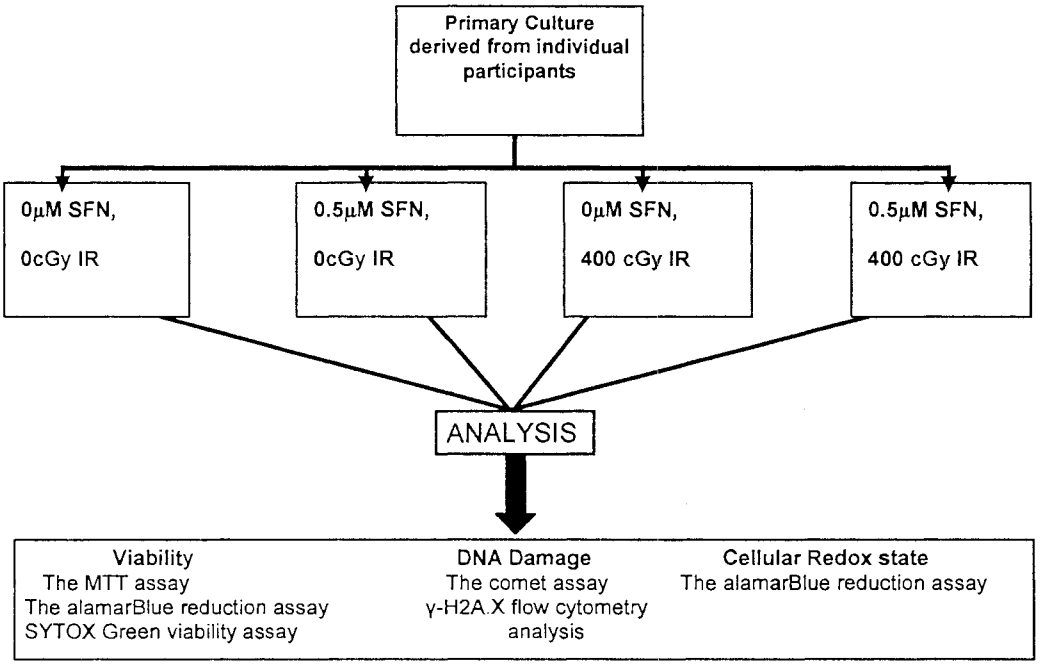


Figure 30: The four experimental conditions and the specific analyses that were completed. The three assays used to detect viability were the MTT assay, the alamarBlue reduction assay, and the SYTOX Green viability assay. The DNA damage detection assays were the comet assay and γ-H2A.X flow cytometry analysis. The alamarBlue assay also measures the change in the reducing environment of the cell, which indicates the cellular redox state.

The MTT assay and the alamarBlue reduction assay were done 24 hours post exposure to ionizing radiation. The SYTOX Green viability assay was done 48 hours post exposure to ionizing radiation. The cells for the comet assay were processed immediately following

radiation exposure. The cells designated for flow cytometry analysis of γ-H2A.X were collected one hour post-radiation to allow for maximum H2A.X phosphorylation, and were then fixed in ethanol. These five assays were done twice for each individual. The results are shown as an average of both replicates, and for all cases the error bars represent the standard deviation from the mean.

4.4.1 Individual 1

This individual was unrelated to other participants in the study. She was 38 years of age, was cancer free and had no family history of breast cancer. Figure 31 A and B are the MTT and alamarBlue results respectively. These analyses were done 24 hours after exposure to radiation. In Figure 31A, the MTT assay showed a decrease in cell proliferation of approximately 10% with each treatment, but results were not significant. A dose of 0.5μM SFN did not offer any protective effects against a radiation dose of 400 cGy. The results of the alamarBlue assay are shown in Figure 31B. This assay is considered an indirect assessment of viability, as well as an assessment of the cellular reducing environment. The results shown in Figure 31B show that there was no difference between treatments for this individual.

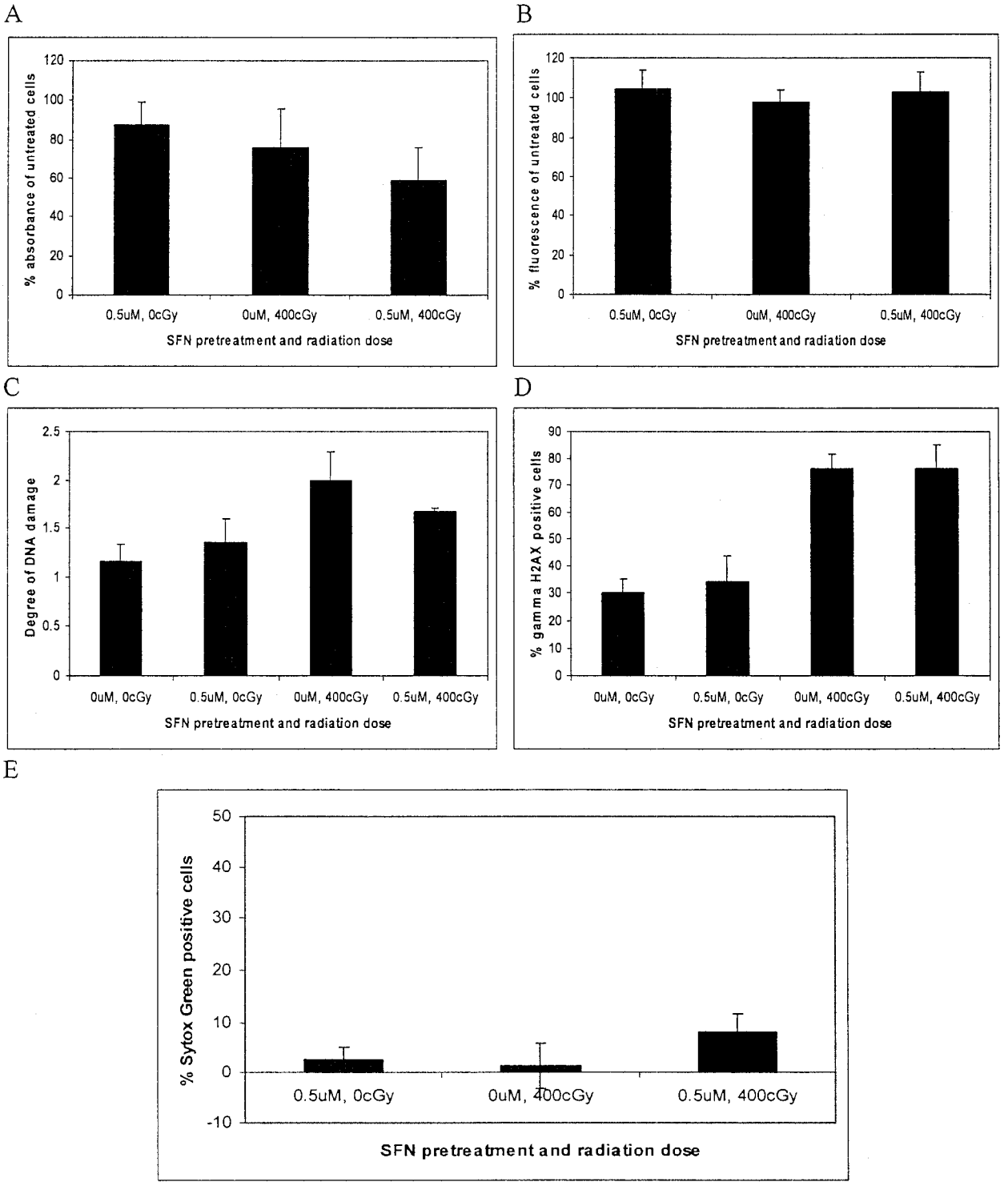


Figure 31: Results of the five experimental assays for Individual 1. **A)** The MTT assay 24 hours after radiation. **B)** The alamarBlue assay 24 hours after radiation. **C)** The comet assay, the slides were prepared immediately after radiation, and 100 comets were scored per slide. **D)** γ-H2A.X flow cytometry analysis, in which cells were fixed in ethanol and stored at -20°C one hour following radiation exposure, 3.0×10^4 cells were analyzed **E)** SYTOX Green viability analysis 48 hours after radiation, 3.0×10^4 cells were analyzed with the flow cytometer.

Figure 31C and D show the results of the comet assay and γ-H2A.X flow cytometry analysis for DNA double strand breaks, respectively. For both assays, there were high levels of background DNA damage. A background level of DNA damage is present because DNA strand breaks can occur during DNA replication in proliferating cells, and some damage can also occur during comet slide preparation. These data are represented numerically in Table 2. The numerical data was generated by calculating the increase in DNA damage above background levels when exposed to ionizing radiation.

Table 2: Numerical data for the DNA damage assays. **A)** The comet assay. **B)** γ-H2A.X flow cytometry analysis.

A) The comet assay

SFN Dose	0cGy Score	400 cGy Score	ΔDNA damage
0μM	1.16	2	0.84
0.5μM	1.355	1.675	0.32

B) γ-H2A.X flow cytometry analysis

SFN Dose	0cGy % γ-H2A.X positive cells	400 cGy % γ-H2A.X positive cells	Δ % γ-H2A.X positive cells
0μM	30.3	76.41	46.11
0.5μM	34.355	76.32	41.985

In the comet assay (Table 2A), the cells treated with SFN alone had a higher level of background DNA damage (1.355) compared to the untreated cells (1.16). However, the increase in general DNA damage induced by radiation exposure in the SFN pretreated cells was smaller (0.32) than the increase in damage of the cells not pretreated with SFN (0.84). The γ-H2A.X flow cytometry results indicate that exposure to ionizing radiation induced significant DNA double strand breaks with or without SFN pretreatment. Therefore, pretreatment with SFN did not reduce levels of DNA double stranded breaks, induced with ionizing radiation.

Figure 31E show the results from the SYTOX Green viability analysis 48 hours after radiation. Interestingly, cells pretreated with SFN, then exposed to ionizing radiation show the

highest level of SYTOX Green positive cells, and therefore, the highest level of membrane disruption. Cells treated with radiation alone, had less membrane disruption than those treated with SFN alone.

4.4.2 Individual 2

This individual was 47 years old and had no personal history of breast cancer, but had a family history of breast cancer and esophageal cancer. She had undergone genetic testing for a BRCA1 mutation and the results were negative. The results for all five experimental analyses are presented in Figure 32.

Figure 32A and B are the MTT and alamarBlue results respectively. The MTT assay (Figure 32A) showed a trend of decreasing mitochondrial dehydrogenase activity when cells are treated with ionizing radiation alone, as well as in the cells pretreated with SFN and radiation, however results were not significant. The alamarBlue assay (Figure 32B) showed no detectable difference between treatments.

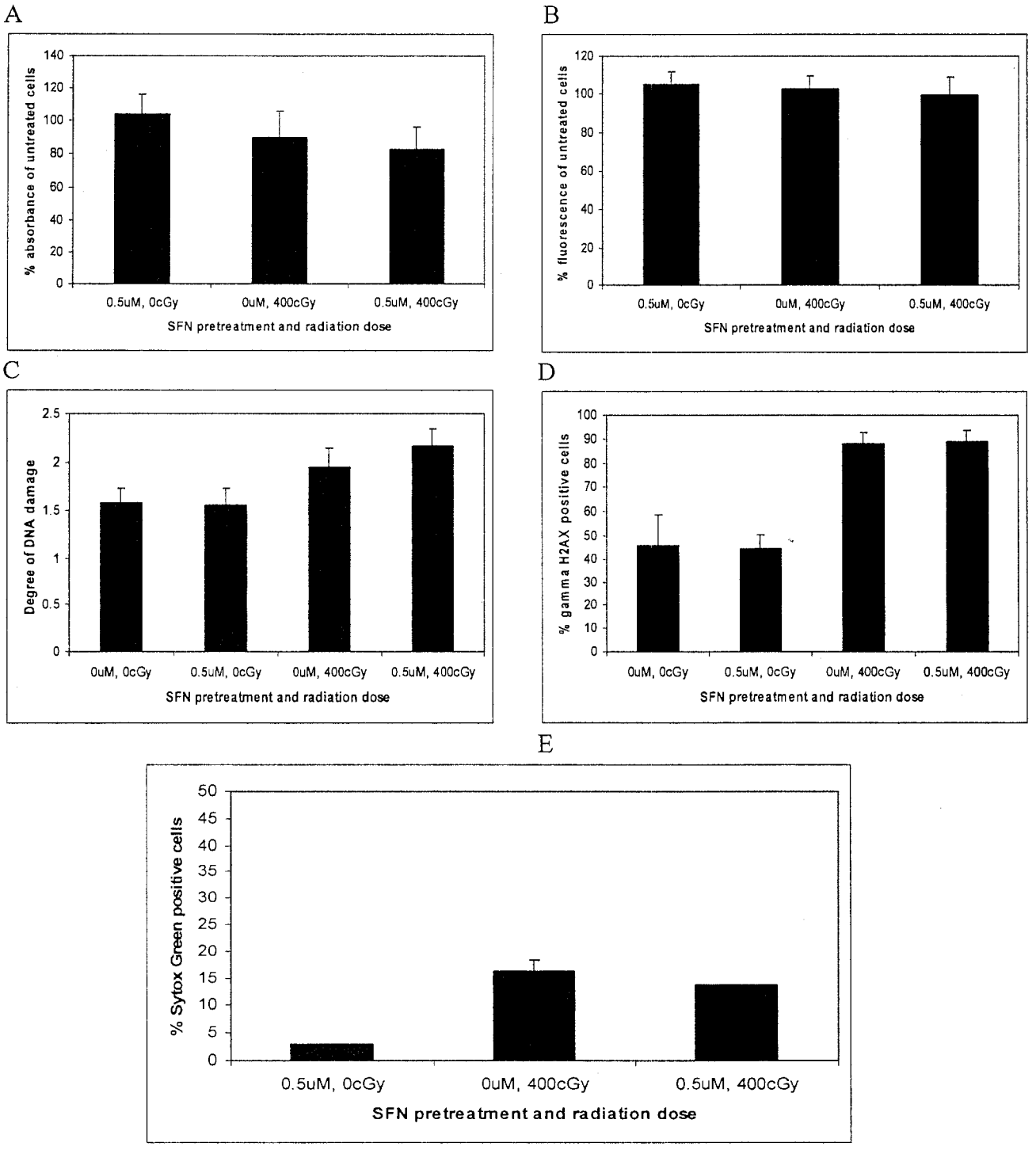


Figure 32: Results of the five experimental assays for Individual 2. **A)** The MTT assay 24 hours after radiation. **B)** The alamarBlue assay 24 hours after radiation. **C)** The comet assay, the slides were prepared immediately after radiation, and 100 comets were scored per slide. **D)** γ-H2A.X flow cytometry analysis, in which cells were fixed in ethanol and stored at -20°C one hour following radiation exposure, 3.0×10^4 cells were analyzed **E)** SYTOX Green viability analysis 48 hours after radiation, 3.0×10^4 cells were analyzed with the flow cytometer.

Figure 32C and D show the results of the comet assay and γ-H2A.X flow cytometry analysis for DNA double strand breaks, respectively. There was a high background level of DNA damage present in these samples and measured in both of these assays. The numerical data are presented in Table 3 A and B.

Table 3: Numerical data for the DNA damage assays. **A)** The comet assay. **B)** γ-H2A.X flow cytometry analysis.

A) The comet assay

SFN Dose	0cGy Score	400 cGy Score	ΔDNA damage
0μM	1.58	1.95	0.37
0.5μM	1.565	2.17	0.605

B) γ-H2A.X flow cytometry analysis

SFN Dose	0cGy % γ-H2A.X positive cells	400 cGy % γ-H2A.X positive cells	Δ % γ-H2A.X positive cells
0μM	45.73	88.025	42.295
0.5μM	44.545	89.115	44.57

In both of these assays, the samples pretreated with SFN alone were not different from the control samples. With the comet assay (Table 3A), the increase in general DNA damage between the cells given SFN was 0.605, compared to the increase in DNA damage in the cells not given SFN, which was 0.37. Similar results were found with γ-H2A.X flow cytometry analysis (Table 3B). The increase of DNA double stranded breaks due to radiation exposure in SFN pretreated cells was not significantly different compared to the cells not given the SFN pretreatment. This indicated the addition of SFN did not decrease DNA damage levels in cells harvested from this individual.

Figure 32E shows the results from a SYTOX Green viability analysis 48 hours after irradiation. The cells treated with SFN showed a small percentage increase in membrane disruption, with only 3% of cells staining positive for SYTOX Green. Cells treated with radiation only showed a relatively high percentage of SYTOX Green positive cells at

approximately 14%. Cells pretreated with SFN and then exposed to ionizing radiation show a comparable level of membrane disruption to that observed in the cells exposed to radiation only.

4.4.3 Individual 3

This individual was 45 years old and had no personal history of breast cancer. She has undergone genetic testing for a BRCA1 mutation and the results were negative. The results for all five experimental analyses are presented in Figure 33.

The MTT results (Figure 33A) for this individual indicated that SFN treatment alone caused a stimulatory effect, and then the general trend is decreasing, but it was they were not significant. The results of the alamarBlue assay show very little difference between treatment groups (Figure 33B).

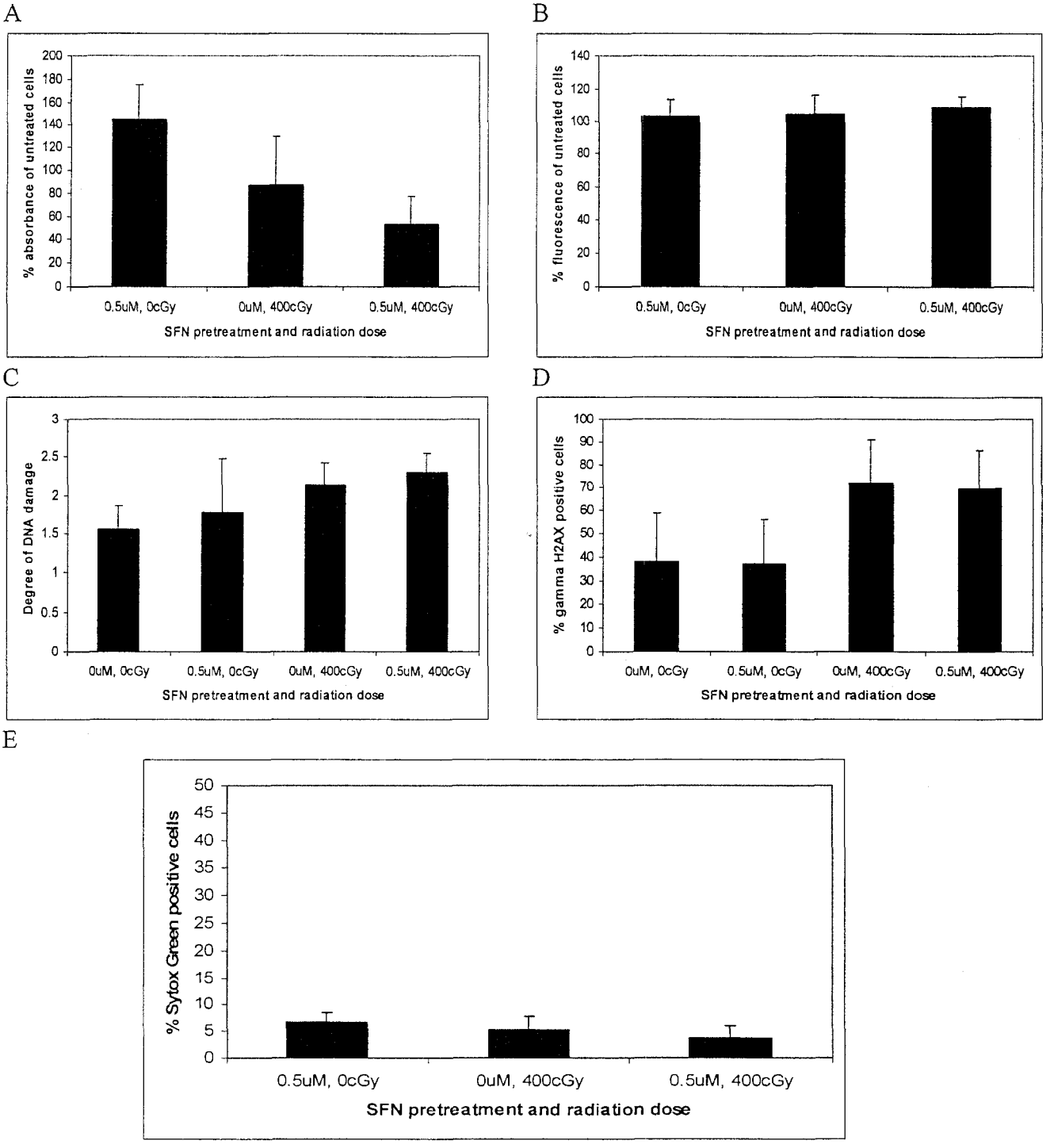


Figure 33: Results of the five experimental assays for Individual 3. **A)** The MTT assay 24 hours after radiation. **B)** The alamarBlue assay 24 hours after radiation. **C)** The comet assay, the slides were prepared immediately after radiation, and 100 comets were scored per slide. **D)** γ-H2A.X flow cytometry analysis, in which cells were fixed in ethanol and stored at -20°C one hour following radiation exposure, 3.0×10^4 cells were analyzed **E)** SYTOX Green viability analysis 48 hours after radiation, 3.0×10^4 cells were analyzed with the flow cytometer.

The DNA damage assays, the comet assay and the γ-H2A.X analysis for DNA double strand breaks, are shown in Figure 33C and D, respectively. Again, the results are represented numerically in Table 4.

Table 4: Numerical data for the DNA damage assays. **A)** The comet assay. **B)** γ-H2A.X flow cytometry analysis.

A) The comet assay

SFN Dose	0cGy Score	400 cGy Score	ΔDNA damage
0μM	1.55	2.135	0.585
0.5μM	1.775	2.305	0.53

B) γ-H2A.X flow cytometry analysis

SFN Dose	0cGy % γ-H2A.X positive cells	400 cGy % γ-H2A.X positive cells	Δ % γ-H2A.X positive cells
0μM	37.91	71.995	34.085
0.5μM	37.095	69.6	32.505

The comet assay results suggested that the presence of SFN did not cause a difference in DNA damage (Table 4A). The γ-H2A.X flow cytometry analysis correlated with the comet assay results, showing that the difference in the increase of γ-H2A.X positive cells after radiation exposure between the two groups is small (Table 4B). The results of these two analyses suggested that SFN did not have any effect on this individual.

Figure 33E shows the SYTOX Green analysis 48 hours post radiation. There were not significant differences between treatment conditions.

4.4.4 Individual 4

This individual was 50 years old and had bilateral breast cancer. The first cancer was diagnosed when she was 42 and then one year later, at 43 received her second diagnosis. She had undergone genetic testing for a BRCA1 mutation and carried a BRCA1germline mutation. She has had previously undergone radiation and chemotherapy. The results for all five experimental analyses are presented in Figure 34.

Figure 34A shows the MTT results for this individual. When treated with radiation alone, there was an approximate 50% decrease in mitochondrial dehydrogenase activity. When given the SFN pretreatment in combination with radiation, there was not a significant increase in enzyme activity. The results for the alamarBue reduction assay are presented in Figure 34B. The results from this assay indicate there was no difference in fluorescence between experimental conditions.

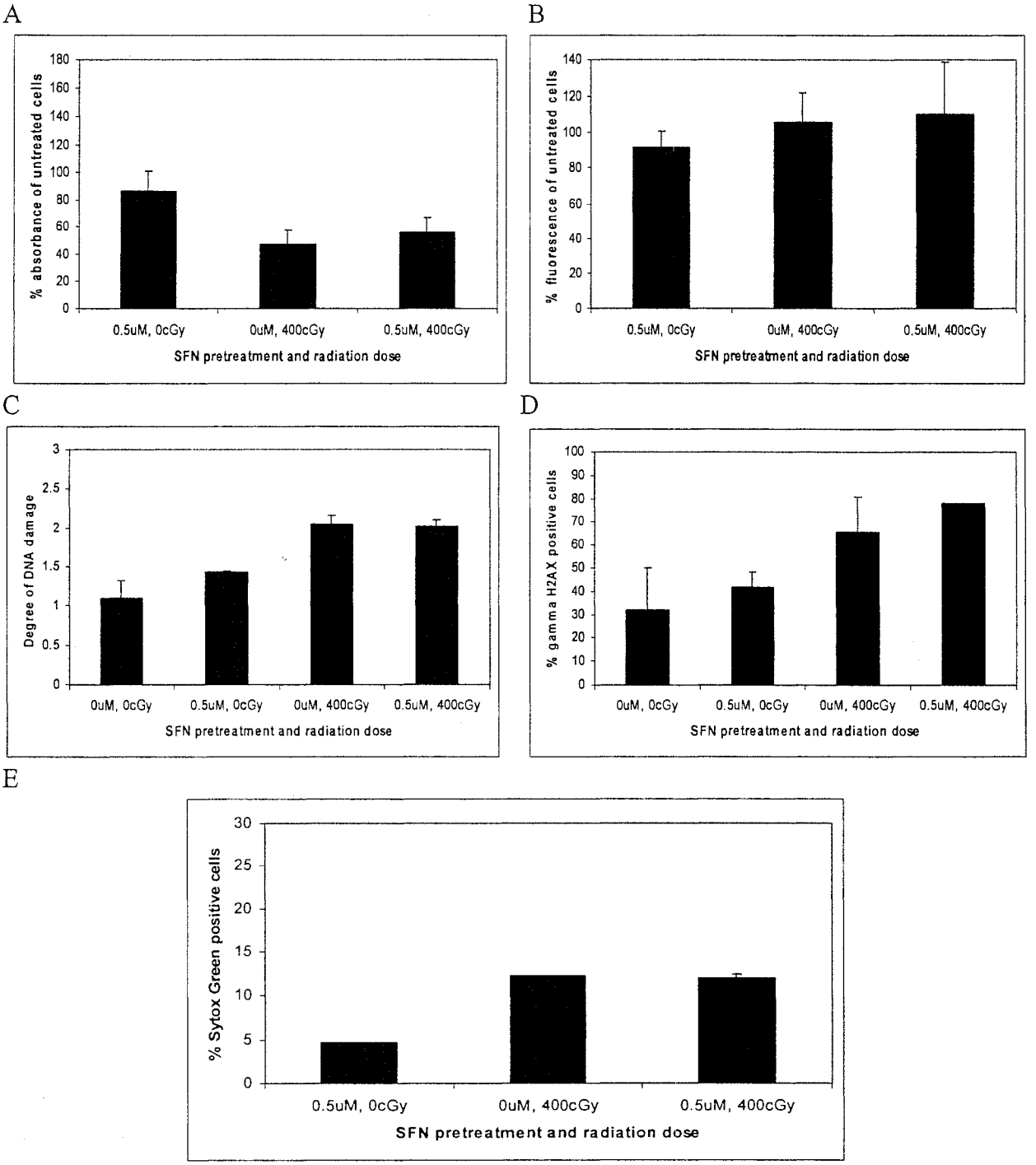


Figure 34: Results of the five assays for Individual 4. **A)** The MTT assay 24 hours after radiation. **B)** The alamarBlue assay 24 hours after radiation. **C)** The comet assay, the slides were prepared immediately after radiation, and 100 comets were scored per slide. **D)** γ-H2A.X flow cytometry analysis, in which cells were fixed in ethanol and stored at -20°C one hour following radiation exposure, 3.0×10^4 cells were analyzed **E)** SYTOX Green viability analysis 48 hours after radiation, 3.0×10^4 cells were analyzed with the flow cytometer.

The results from the DNA damage assays are presented in Figure 34 C and D. This data is represented numerically in Table 5. For both assays, treatment with radiation caused a significant increase in DNA damage. The addition of SFN prior to irradiation exposure did not alleviate levels of the induced damage detected with both the comet assay and γ-H2A.X flow cytometry analysis.

Table 5: Numerical data for the DNA damage assays. **A)** The comet assay. **B)** γ-H2A.X flow cytometry analysis.

A) The comet assay

SFN Dose	0cGy Score	400 cGy Score	ΔDNA damage
0μM	1.11	2.05	0.94
0.5μM	1.43	2.025	0.595

B) γ-H2A.X flow cytometry analysis

SFN Dose	0cGy % γ-H2A.X positive cells	400 cGy % γ-H2A.X positive cells	Δ % γ-H2A.X positive cells
0μM	32.105	65.38	33.275
0.5μM	41.705	78.16	36.455

Figure 34E shows the SYTOX Green data collected 48 hours after cells underwent radiation exposure. The level of SYTOX Green positive cells are comparable, at approximately 12%, for both samples, with or without the SFN pretreatment, that were exposed to ionizing radiation.

4.4.5 Individual 5

This individual was 44 years old, had breast cancer and was treated with chemotherapy. She was a carrier of a BRCA1 germline mutation. The results for all five experimental analyses are presented in Figure 35.

From the MTT assay (Figure 35A), cells pretreated with SFN alone show an increase in mitochondrial dehydrogenase activity of approximately 28% above normal. When exposed to ionizing radiation alone, there is large decrease in this activity of approximately 50%. The decrease was comparable in cells that were pretreated with SFN followed by ionizing radiation. The alamarBlue assay showed no difference in fluorescence between treatment groups (Figure 35B).

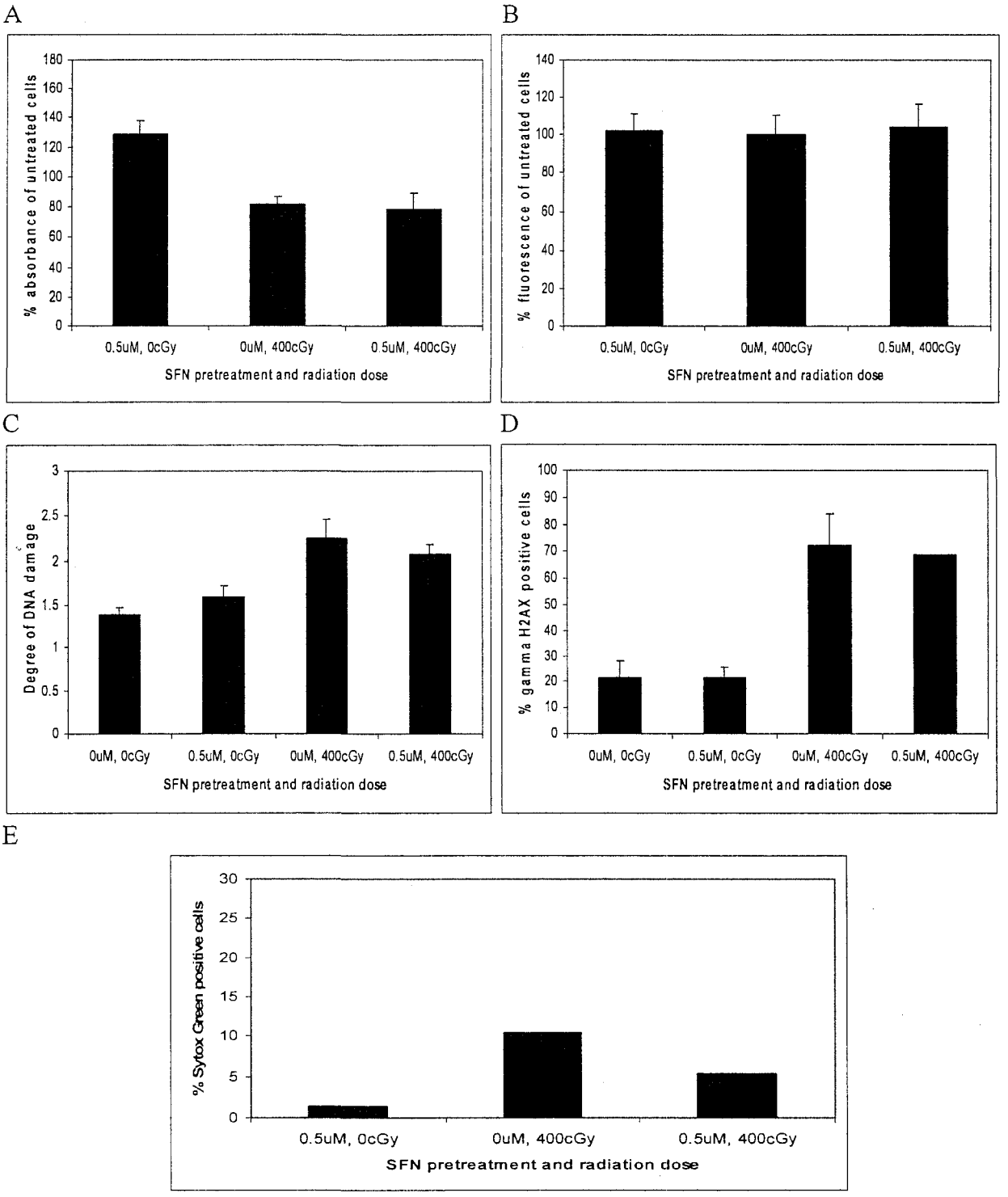


Figure 35: Results of the five assays for Individual 5. **A)** The MTT assay 24 hours after radiation. **B)** The alamarBlue assay 24 hours after radiation. **C)** The comet assay, the slides were prepared immediately after radiation, and 100 comets were scored per slide. **D)** γ-H2A.X flow cytometry analysis, in which cells were fixed in ethanol and stored at -20°C one hour following radiation exposure, 3.0×10^4 cells were analyzed **E)** SYTOX Green viability analysis 48 hours after radiation, 3.0×10^4 cells were analyzed with the flow cytometer.

For the DNA damage assays (Figure 35 C and D), the numerical data is shown in Table 6. According to the comet assay, cells pretreated with SFN had an increase of 0.485 in general DNA damage after radiation exposure (Table 6A). Similar results were obtained with γ-H2A.X flow cytometry analysis (Table 6B). Without the SFN pretreatment, there was a significant increase in the percentage of γ-H2A.X positive cells after radiation (51.05%). The addition of SFN prior to irradiation did not alleviate levels of the induced damage detected with both the comet assay and γ-H2A.X flow cytometry analysis.

Table 6: Numerical data for the DNA damage assays. **A)** The comet assay. **B)** γ-H2A.X flow cytometry analysis.

A) The comet assay

SFN Dose	**0cGy Score**	**400 cGy Score**	**ΔDNA damage**
0μM	1.385	2.26	0.875
0.5μM	1.595	2.08	0.485

B) γ-H2A.X flow cytometry analysis

SFN Dose	**0cGy % γ-H2A.X positive cells**	**400 cGy % γ-H2A.X positive cells**	**Δ % γ-H2A.X positive cells**
0μM	21.665	72.705	51.04
0.5μM	21655	68.77	47.115

SYTOX Green analysis 48 hours after ionizing radiation exposure (Figure 35E) showed that there was a loss of membrane integrity associated with the dose of ionizing radiation alone. It also appears that the addition of SFN prior to irradiation decreased the loss of membrane integrity of by approximately 5%.

4.5 Combined individual results for all assays

Table 7 is a summary table of the participants describing their age, BRCA1 status, cancer occurrence, treatment history, as well as relationship to other participants in this study.

Table 7: Summary of breast cancer history and BRCA1 status for each study participant.

Individual	Age	BRCA1 mutation status	Cancer history	Radiation therapy	Chemo-therapy	Relationship to other participants
1	38	-	-	N/A	N/A	none
2	47	-	-	N/A	N/A	Sister to 3,4,5
3	45	-	-	N/A	N/A	Sister to 2,4,5
4	50	+	+	+	+	Sister to 2,3,5
5	44	+	+	-	+	Sister to 2,3,4

The results for the MTT assay are presented in Figure 36. Individuals 4 and 5 both had significant decrease in cell proliferation when treated with radiation alone, as measured by the MTT assay. This result was deemed significant as the standard deviation error bars did not overlap. Both these individuals are positive for a BRCA1 germline mutation, and had breast cancer. The addition of the SFN pretreatment did not offer protection against the ionizing radiation as indicated by an analysis of results from the MTT assay.

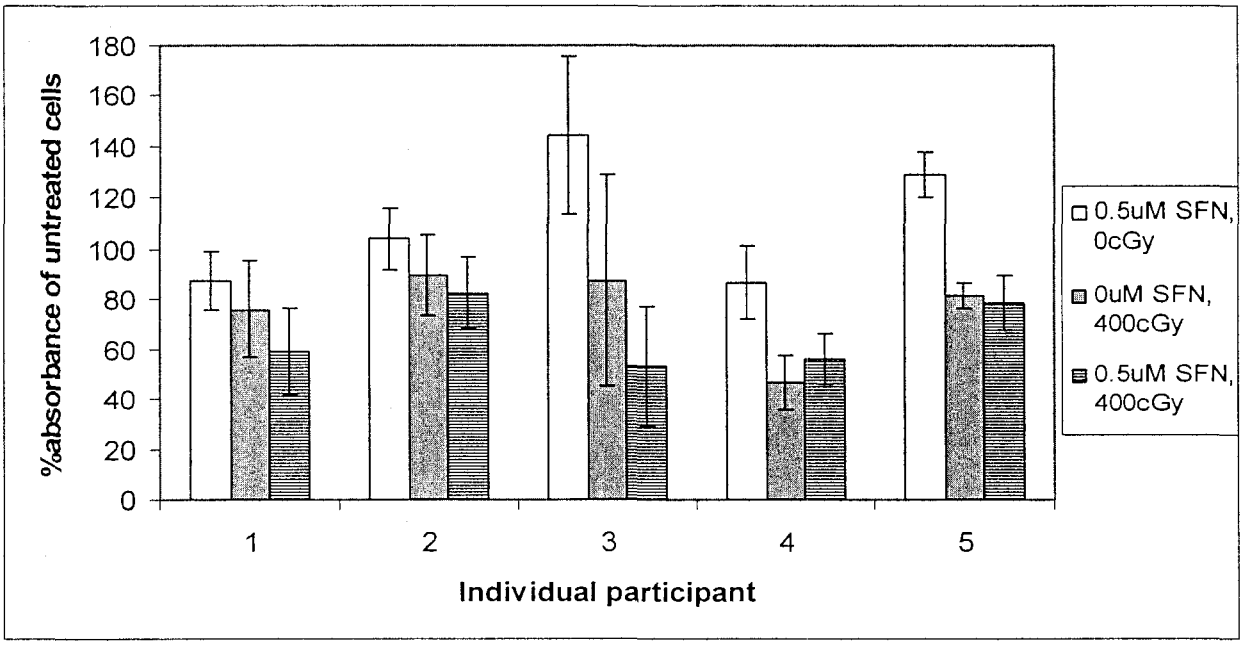


Figure 36: The combined MTT results for all individuals

The combined results for the alamarBlue assay are presented in Figure 37. The results of the alamarBlue assay indicated that there were no significant difference between treatment groups, or between specific individuals.

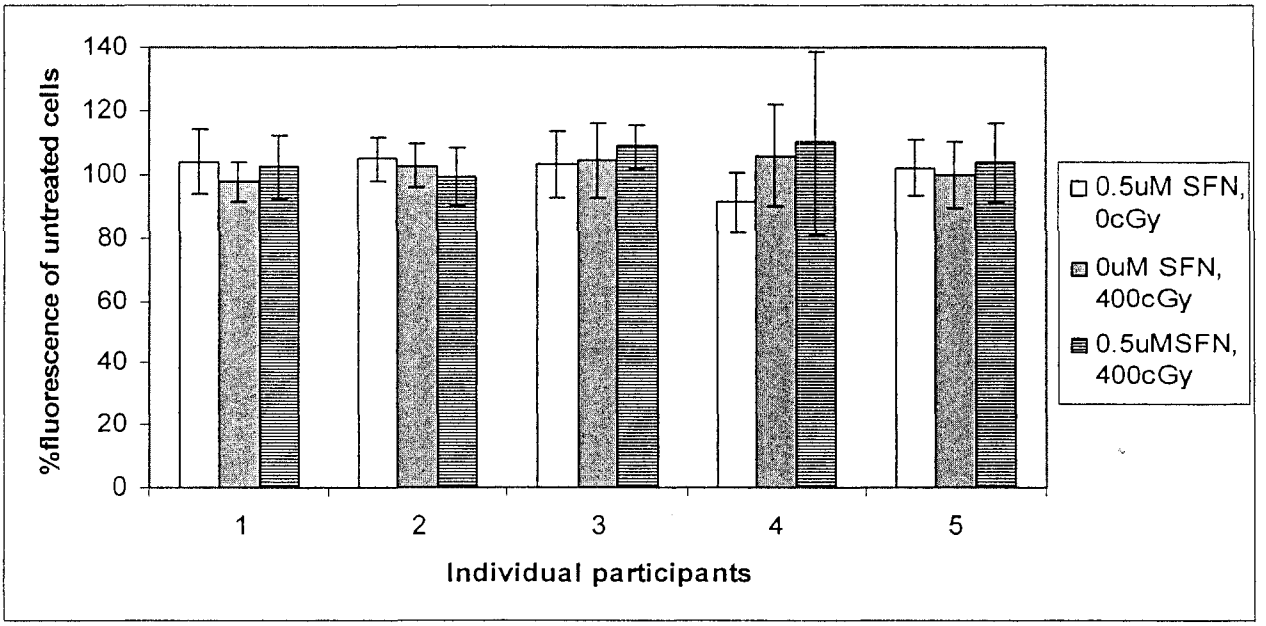


Figure 37: The combined alamarBlue results for all individuals

The comet assay results show that ionizing radiation caused an increase in DNA damage for all individuals (Figure 38). The addition of the SFN pretreatment did not correspond with a decrease in damage caused by ionizing radiation. These results show that pretreatment with SFN was not effective in decreasing the level of DNA damage after radiation treatment.

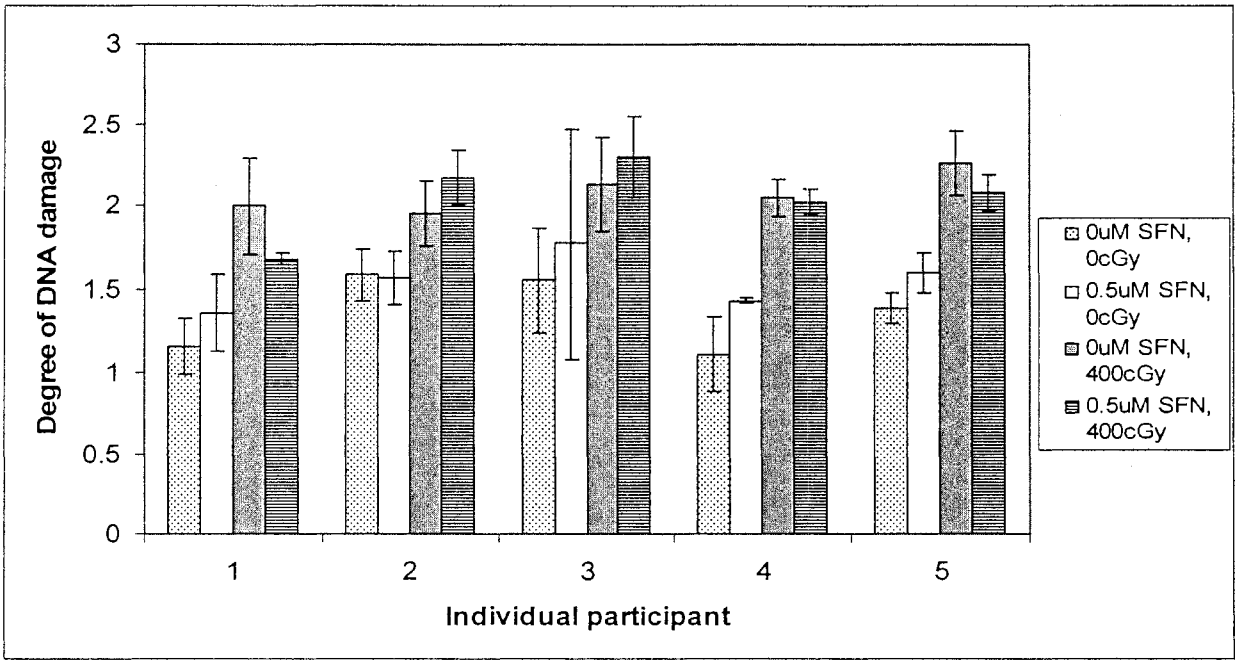


Figure 38: The combined comet assay results for all individuals

The combined results for the γ-H2A.X flow cytometry analysis (Figure 39) also indicated that radiation caused an increase in γ-H2A.X positive cells. Individual 5 shows the largest increase in γ-H2A.X positive cells between background and treated samples. These results indicated the addition of the SFN pretreatment did not greatly reduce the levels of γ-H2A.X positive cells. These results also indicated that Individual 5, who carried a BRCA1 mutation, was more sensitive to radiation.

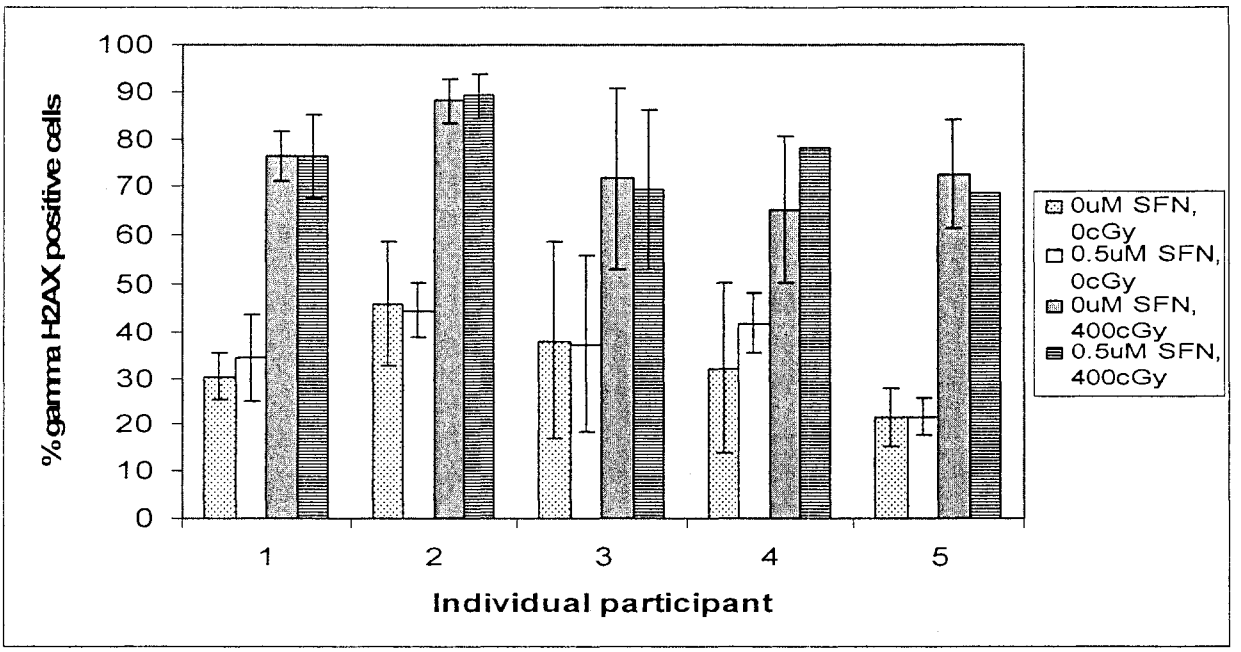


Figure 39: The combined γ-H2A.X results for all individuals

The combined results of the SYTOX Green assay are presented in Figure 40. Generally, ionizing radiation does caused an increase in SYTOX Green positive cells, except in the case of Individual 3 (related control, no BRCA1 mutation). In the case of Individual 1, the addition of the SFN pretreatment caused an increase in the percentage of SYTOX Green positive cells. In the case of Individuals 2, and 5, the addition of SFN corresponds to a decrease in SYTOX Green positive staining cells.

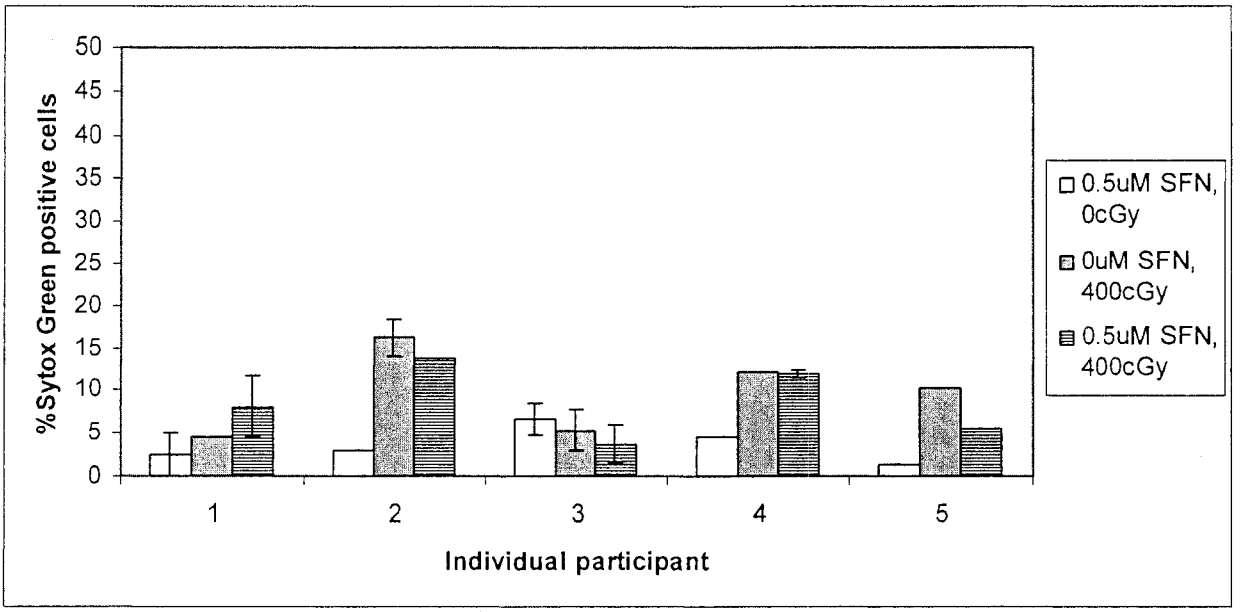


Figure 40: The combined SYTOX Green results for all individuals. Error bars are missing in some cases due to lack of sample.

5. Discussion

5.1 Effect of ionizing radiation on the redox status of MCF-7 cells

Ionizing radiation induces oxidative stress through the production of ROS. Antioxidants, both enzymatic and non-enzymatic, neutralize the ROS by breaking them down before they can damage the critical target, which is DNA. Increased GSH levels are important in generating resistance to oxidative stress induced by ionizing radiation in cells (Meister 1988).

Glutathione is a tripeptide of γ-glutamate, cysteine and glycine and is one of the major reductants found in eukaryotic cells (Dumitrescu and Cotarla 2005; McEligot et al. 2005). This low molecular weight thiol exists predominantly in the reduced form in the cytosol, mitochondria and nucleus; and therefore protects the cell from the reactive oxygen species (ROS) produced during normal metabolism (Valko et al. 2007). One of the major indicators of the redox status of a cell is the GSH/GSSG system. In the presence of ROS, and with the help of other enzymes such as glutathione peroxidase, GSH is converted to the oxidized form, glutathione disulphide (GSSG). The redox ratio decreases as the concentration of GSSG increases. An elevated concentration of GSSG leads to the activation of various signaling pathways (for example, MAPK- and NF-κB- mediated signaling pathways) that reduce cell proliferation and trigger apoptosis (Valko et al. 2007; Wu et al. 2004).

SFN is an isothiocyanate (ITC), which is a large planar molecule containing a thiol group. SFN is the most potent naturally-occurring inducer of the phase II enzymes that is known, and it operates through activation of the antioxidant response element (ARE) (Nguyen et al. 2003). Proteins encoded by the ARE include enzymes associated with glutathione biosynthesis and the Phase II enzymes (Nguyen et al. 2003). Phase II enzymes include glutathione-S-transferases and NAD(P)H:quinine oxidoreductases (NQO1) which detoxify

electrophiles and ROS by several mechanisms and therefore promote their quenching (Fimognari et al. 2005). Glutathione-S-transferases (GSTs) initiate the reaction of GSH with electrophiles and physiological metabolites, such as estrogen, to begin detoxification (Wu et al. 2004). A study done with murine hepatoma cells demonstrated that SFN accumulation in the cells is dependent on cellular GSH levels, as the SFN becomes conjugated to the GSH (Zhang 2000). A subsequent study done by the same group, found that GST enhances the conjugation reaction between SFN and GSH (Zhang 2001). SFN has been previously shown to be an important factor in the regulation of redox status through the induction of thioredoxin reductase in human breast cancer cells (Wang et al. 2005).

The results seen in Figure 12 show that ionizing radiation exposure depletes the cellular GSH level in MCF-7 cells. Radiation exposure induces high levels of ROS and therefore, the GSH is converted to GSSG, causing a decrease in the redox ratio. The MCF-7 cells that were pre-treated with SFN show an increased redox ratio. These results indicate that SFN may be activating the ARE, and therefore inducing expression of glutathione transferases (a phase II enzyme), as well as enzymes important in the biosynthesis of GSH. Although these cells were exposed to ionizing radiation, the addition of SFN protects against the depletion of the reduced GSH stock, and enables the cell to maintain an elevated redox ratio.

5.2 Technical limitations of using non-proliferating lymphocytes

Lymphocytes are an alternative model system to immortalized cell lines. Currently, many studies on the radiosensitivity and chemosensitivity of cells with BRCA1 mutations have been limited to established cell lines that have relatively high proliferation rates, to further understand how these mutations lead to carcinogenesis. The study done by Bae and colleagues(2004), investigated the effects of BRCA1 overexpression, underexpression and

mutational activation in mouse embryonic fibroblast (MEF) cells as well as prostate, breast and lymphoblastoid cancer cell lines (Bae et al. 2004). Primary cell cultures derived from patients would presumably have lower metabolic activity, and would therefore better represent the response to ionizing radiation of a normal, non-transformed cell in the body. Immortalized cell lines derived from peripheral blood lymphocytes, such as lymphoblastoid cell lines, show very different responses to ionizing radiation and cytotoxic agents than primary lymphocytes from blood (Trenz et al. 2005). Lymphocytes are easily obtained and can be easily maintained in tissue culture conditions compared to cells obtained from tumour tissue. Recent studies compared ascites cells to lymphocytes obtained from peripheral blood. These ascites cells derived from the peritoneal cavity of cancer patients with metastatic disease were very difficult to obtain and to maintain in tissue culture conditions (Richard et al. 2005). The novelty of this study is in using primary lymphocytes derived from individuals with a known heterozygous BRCA1 mutation, and in analyzing the response of these cells to ionizing radiation.

Non-proliferating lymphocytes were used initially because they best represent differentiated cells in the body, similar to those that might inadvertently be impacted by radiation exposure during radiation therapy. Also, previous studies have shown that stimulating agents such as PHA and IL-2 confer radiation resistance in primary lymphocytes and can change their behavior in response to other genotoxic agents (Brazowski et al. 2007; Ishioka et al. 1997; Lavin and Kidson 1977). This could be because enzymes needed for DNA replication also function in DNA repair, such as DNA ligases (Ellenberger and Tomkinson 2008). The expression of these enzymes is upregulated in proliferating cells (Stedeford et al. 2001). By stimulating proliferation of the lymphocyte cultures, these enzymes may be more readily available to repair the damage caused by radiation exposure. This was evident in Figure 13, which depicted a 70% increase in

γ-H2A.X positive cells after ionizing radiation exposure, compared to actively proliferating cells, which showed an increase of only 20%. The non-proliferating lymphocyte culture showed a striking increase in γ-H2A.X positive cells after exposure to ionizing radiation. Based on the results of this experiment, further experiments using non-proliferating lymphocytes were pursued.

The major technical issues encountered while working with the non-proliferating lymphocyte cultures was the small percentage of CD3 positive cells that were present in the culture and the short (five day) length of time this population survived in tissue culture conditions (Figure 14). The inadequate T-cell population limited the type and the number of experiments that could be done with this cell population. For example, 1.0×10^6 cells were needed to obtain a detectable colour change by spectrophotometer in the glutathione assay, in order to calculate the total micromolar concentration of intracellular glutathione (Figure 17). The heterogeneous cell population as well as the increasing levels of debris as time in culture progressed also made it difficult to assess the true cytoxicity of SFN in these primary cultures (Figure 15).

A radiation dose response curve was successfully generated for non-proliferating lymphocyte cultures using the SYTOX Green viability assay (Figure 16). Analyses that were done one hour after radiation exposure indicated no increase in the percentage of SYTOX Green positive cells, even at the highest dose of radiation used (1000cGy) (Figure 16A). Analyses that were done 24 hours post radiation exposure showed a steady increase in SYTOX Green positive cells as the radiation dose increased (Figure 16B). The increase in SYTOX Green positive cells indicated that there was an increase in cell death, as loss of membrane integrity is a characteristic

of both necrosis and apoptosis (Fiers et al. 1999; Vanden Berghe et al. 2004). It was found that a radiation dose of 200 cGy decreased cell viability by approximately 15%.

Based on the previous experimental results that were obtained using γ-H2A.X analysis (Figure 13), cells pretreated with a 10μM dose of SFN prior to ionizing radiation exposure were analyzed (Figure 18). However, the division of the limited cell population into four experimental groups (refer to Figure 30) markedly decreased the number of cells available in each sample, and this problem was further compounded by loss of cells during sample preparation. Initial results had indicated that the SFN pretreatment reduced the level of DNA double strand breaks (Figure 18), but based on the limited availability of cells, a valid data analysis could not be performed for this experiment.

The heterogeneous cell population found in non-proliferating lymphocyte cultures consistently lead to inconclusive experimental results, and the lack of adequate cell numbers limited the types of possible experiments that could be done using lymphocytes that were not stimulated to proliferate.

5.3 Optimization of techniques using proliferating lymphocytes

Even though stimulating proliferation in lymphocyte cultures confers some radiation resistance, the technical difficulties associated with using non-proliferating lymphocyte cultures made it necessary to consider using stimulating agents to induce proliferation in the lymphocyte cultures in order to obtain a homogeneous cell population of adequate size. In Figure 13, the increase in γ-H2A.X positive cells in the proliferating culture after exposure to ionizing radiation is not nearly as striking as that seen in the non-proliferating culture; however, there was still a significant 20% increase of γ-H2A.X positive cells above the background level in proliferating lymphocytes (Figure 13B). It has been recently shown that there is an incidence of γ-H2A.X foci

that are not involved in DNA double strand break repair. This population of small γ-H2A.X nuclear foci can be detected using immunocytochemical techniques. These small, γ-H2A.X foci do not interact with DNA repair proteins, and occur independently of DNA double strand break induction (McManus and Hendzel 2005). Under normal growth conditions, the number of these small γ-H2A.X foci are at the highest level during the G_2/M phase of the cell cycle (McManus and Hendzel 2005). Normal growth conditions refer to cycling cells that are not in contact with genotoxic or cytotoxic agents. The exact function of the small γ-H2A.X foci is still unknown, but one hypothesis is that they may be involved in the mitotic process (McManus and Hendzel 2005). These small γ –H2A.X foci could significantly contribute to the high background level of γ-H2A.X positive cells seen in the stimulated and proliferating lymphocyte culture (Figure 13B). However, these analyses were performed using flow cytometry which measures the overall fluorescence intensity of a cell population. To assess the levels of small versus large γ-H2A.X foci, it would be necessary to perform immunofluorescent staining of cells grown on coverslips and analyzed by fluorescence microscopy.

There were obvious advantages to using proliferating lymphocyte cultures for this study. In contrast to non-proliferating cells, the percentage of CD3 positive cells in proliferating culture was approximately 85% until at least day 9 in culture, and this percentage was consistent between individuals (Figure 19). This allowed for optimization of techniques, as multiple experiments were able to be performed with the cells from a single blood sample. The techniques used in this study were the MTT assay, the alamarBlue reduction assay, SYTOX Green analysis, the comet assay, and γ-H2A.X flow cytometric analysis.

Using the MTT and alamarBlue assays (Figures 22 and 23), it was determined that a dose of 0.5 μM SFN would not be cytotoxic to the proliferating lymphocyte culture.

SFN was toxic to proliferating lymphocytes at lower concentrations compared to immortalized cell lines. Using lymphocytes, a dose range of 0 to 10 μM SFN was used for the MTT and alamarBlue assays, with 10μM being cytotoxic to approximately 95% of the cell population, as determined by both assays. Further evidence that cell lines respond to cytotoxic reagents differently than primary cells is presented in Figure 10B. The MTT results for MCF-7 cells indicate that a dose of SFN as high as 20 μM would not significantly reduce viability. These experiments support the rationale for using primary lymphocyte cultures rather than immortalized breast cancer cell lines. Other studies have produced similar results, showing that immortalized cell lines exhibit very different responses to cytotoxic agents as compared to primary cell cultures (Richard et al. 2005)

Additional experiments (data not shown) were done using the paired cell lines, HCC 1937 and HCC 1937 BL. The paired cell lines are derived from the same 24-year-old female patient and were acquired from the American Type Culture Collection. The HCC1937 cell line is homozygous for the BRCA1 5382C mutation and is derived from a primary ductal carcinoma. HCC1937 BL is heterozygous for the BRCA1 5382C mutation and is a B lymphoblastoid cell line derived from peripheral blood lymphocytes and transformed using Epstein-Barr virus. Using these cell lines, it determined that the LD_{50} of the HCC1937 BL cell line was approximately 17μM; however, a dose as high as 100μM did not show toxic effects in the HCC1937 cell line (data not shown). These data provide additional evidence that tumour-derived cells exhibit very different responses to cytotoxic agents than do non-tumour-derived cells.

From the results of the SYTOX Green viability assay, the MTT assay, and the alamarBlue reduction assay, a radiation dose of 400 cGy was determined to be sufficient to induce detectable levels of cell damage in proliferating lymphocytes (Figures 25-27).

Results of the MTT assay showed radiation doses at the lower end (under 200 cGy), caused a hormetic effect, as seen, for example, at a radiation dose of 25cGy in Figure 26. Hormesis is defined as the stimulating effect of small doses of substances of which large doses are toxic. At 400 cGy, there was an approximate reduction in cell viability to 60%, indicating that this dose of radiation was toxic for 40% of the population. At the maximum dose tested, 1000cGy, approximately only 40% of cells remained viable.

The alamarBlue reduction assay (Figure 27), did not detect the same decrease in cell proliferation as the MTT assay at the higher doses of ionizing radiation tested. The decrease in cell viability as measured by the alamarBlue assay was approximately 35-40% at 400 cGy, and this value remained constant as the dose increased to the maximum applied dose of 1000cGy. These results highlight that both the MTT and alamarBlue assays measure viability indirectly and by different means.

The SYTOX Green viability analysis with proliferating lymphocytes (Figure 24) showed that there was not a detectable increase in cell death, as measured by membrane disruption, one hour after radiation exposure. This is consistent with results of the SYTOX Green assay obtained using non-proliferating cells (Figure 16A). When proliferating cells were given a radiation dose of 400 cGy there was an increase in the SYTOX Green positive cell population (damaged cells) of approximately 10%, 24 hours after radiation exposure (Figure 25). In this proliferating cell population, approximately 18% were SYTOX Green positive at the maximum radiation dose of 1000cGy. However, in the non-proliferating population (Figure 16B),

approximately 28% of cells were SYTOX Green positive at the maximum dose of 1000cGy. Similar to the original γ-H2A.X experiment (Figure 13), this indicates that treating lymphocyte cultures with proliferating reagents such as PHA and IL-2, desensitizes lymphocytes to ionizing radiation, which concurs with results found in published studies that have used irradiated proliferating lymphocytes (Lavin and Kidson 1977).

Radiation dose response curves were also generated using the HCC 1937 BL cell line (data not shown). This cell line is relatively radiation resistant, when compared to proliferating lymphocytes. Even at the highest dose of radiation applied (1000cGy), the alamarBlue and MTT assays both showed a decrease in viability of less than 20%. Similar results were obtained using SYTOX Green analysis, where there was only an increase in damaged cells of approximately 8%, 24 hours after exposure to a radiation dose of 1000cGy. These results further demonstrate that lymphoblastoid cell lines respond to ionizing radiation differently compared to primary blood cells, and further support the use of primary lymphocytes for this study.

The two DNA damage assays performed, the comet assay (Figure 29) and the γ-H2A.X flow cytometry assay (Figure 28), detected a significant amount of DNA damage after the proliferating lymphocytes were exposed to a radiation dose of 400 cGy. It is important to differentiate between the types of DNA damage detected by each assay. The comet assay was used to detect the overall level of DNA damage induced by the ionizing radiation. The alkaline comet assay was used in this study because it is the most sensitive comet assay as it detects all types of DNA damage (Olive et al. 1992). These include alkali labile sites, single strand breaks, double strand breaks, DNA cross-links and incomplete excision repair sites (Colleu-Durel et al. 2001; Trenz et al. 2005). In contrast, the neutral comet assay only detects DNA double strand breaks and cross-links (Trenz et al. 2005). Single strand breaks and other small lesions, such as

base modification, are easily repaired and do not contribute to cell death caused by ionizing radiation (Ward 1995). The purpose of this assay was to determine the amount of overall DNA damage induced through ionizing radiation exposure.

The γ-H2A.X flow cytometry assay specifically detects the presence of DNA double strand breaks, which is the most critical lesion that can occur in DNA in response to ionizing radiation. The generation of double strand breaks is beneficial when ionizing radiation is used to treat a tumor during cancer therapy, as the induction of multiple double strand breaks overloads the cancerous cell's DNA repair capacity, resulting in cell death (Wang et al. 2001). However, when occurring in normal healthy cells, and if not repaired properly, DNA double strand breaks can lead to cell death or the induction of mutations and gene translocations which may eventually lead to cancer (Bau et al. 2006).

There are two possible pathways for DNA double strand break repair: non-homologous end joining (NHEJ) and homologous recombination (HR) (Gudmundsdottir and Ashworth 2006). NHEJ is an error-prone repair pathway, because at the site of the break, the joining of previously unlinked nucleotides occurs, resulting in minor changes in the DNA sequence (Gudmundsdottir and Ashworth 2006). These minor changes can eventually result in gross chromosomal rearrangements which lead to genomic instability (Turner et al. 2005). Interestingly, in BRCA1 deficient cells, error-prone NHEJ predominates as the major mechanism for DNA double strand break repair (Venkitaraman 2002).

The second repair pathway, HR, uses a homologous sequence, such as a sister chromatid, as the template to synthesize the correct DNA sequence at the site of the double strand break (Gudmundsdottir and Ashworth 2006). Upon DNA double strand break induction, BRCA1 is one of the first proteins to localize to γ-H2A.X foci, and this indicates that it may be important in

cell signaling pathways and the recruitment of other DNA repair proteins, such as RAD51 (Venkitaraman 2002). While the exact role of BRCA1 in DNA repair is still unclear, there is much evidence to support its role in both DNA double strand break repair pathways, which emphasize its importance in the cellular response to ionizing radiation.

Using actively proliferating lymphocyte cultures allowed for experiments to be completed using a homogeneous, CD3-positive cell population that persisted for at least nine days in culture conditions (Figure 20). Through cell viability and DNA detection assays, it was determined that when working with the patient samples, the SFN pretreatment would be 0.5μM, and a radiation dose of 400 cGy would be optimal to study the cellular response of ionizing radiation and SFN on cell viability, redox potential and DNA damage.

5.4 Description of study participants

Individuals 2,3,4, and 5 are female members (sisters) of the same family (Figure 7), which has a compelling history of cancer prevalence. The following discussion will explain the relevance of this family's genetic pedigree, what is known about the specific germline BRCA1 mutation that is carried by this family, and the clinical relevance of this mutation. Additionally, the cancer treatment plans for both Individuals 4 and 5 will be discussed. Individual 1 served as an age matched control with no family or personal history of breast cancer.

The family pedigree presented in Figure 7 indicates that Individuals 2, 3, 4 and 5 had family members on both the maternal and paternal sides who were afflicted with breast cancer. Their aunt on the paternal side passed away at the age of 53 from the disease. Their mother was diagnosed with breast cancer at age 35, and she passed away at age 38 from her disease. The results of genetic testing for BRCA1 mutations determined that the germline mutation in this family is maternally inherited, as the father of Individuals 2-5 underwent genetic testing and is

not a carrier of the mutation found in the next generation (Individuals 4 and 5). Early onset breast cancer, in which diagnosis of the disease occurs before the age of 40, is frequently associated with BRCA1 germline mutations (Dite *et al* 2003; Krainer *et al* 1997). Individuals carrying a germline BRCA1 mutation have a 20% chance of being diagnosed by the age of 40 and an 80% chance by the age of 70, indicating that the penetrance of the gene increases with age (Krainer 1997). Penetrance refers to the likelihood that a mutation will induce a clinical phenotype in the individual (Weinberg 2007).

As previously mentioned, the specific BRCA1 mutation found in this family, c.5074+3A>G, is very rare. It was previously documented in the Netherlands by Dr. A. van der Hout, and this is the only incidence of this specific mutation recorded in the Breast Cancer Information Core (BIC) database. The BIC database is part of the National Human Genome Research Institute at the National Institutes of Health. According to the BIC database the clinical relevance of this mutation is classified as unknown. However, in this report coming from the Netherlands, this mutation disturbs the proper splicing of the *BRCA1* pre-mRNA, which leads to the skipping of exon 17 and was found to be clinically relevant (A.H. van der Hout 2007). The pedigree of the family highlighted in this study strongly suggests that this mutation has clinical relevance. The mother of Individuals 2, 3, 4, and 5 was diagnosed with breast cancer at age 35, and her daughter, Individual 5, was diagnosed at age 38. Another daughter, Individual 4, was diagnosed with bilateral breast cancer at age 42, and Individual 4 also has two daughters who carry this same germline mutation. One daughter of Individual 4 was diagnosed with breast cancer at age 23 and the other daughter is presently presymptomatic at age 28. Individuals 2 and 3 are not carriers of the germline BRCA1 mutation and have no personal incidence of breast cancer.

As mentioned, Individual 4 was diagnosed with bilateral breast cancer at age 42. Her treatment plan included bilateral mastectomies and chemotherapy, as well as radiation therapy. Chemotherapy and radiation therapy both generate ROS to induce cytotoxicity in the tumor cells (Blasiak et al. 2004). Individual 4's chemotherapy regimen consisted of CEF (Cyclophosphamide, Epirubicin, and Fluorouracil) over a period of five months. Cyclophosphamide is alkylating agent which will slow the growth of cancer cells. Epirubicin is an anthracycline drug that intercalates into DNA to interfere with replication, and it also induces free radical mediated cell death (Fedier et al. 2003). Fluoruracil blocks synthesis of thymidine, and therefore interferes with DNA replication. Individual 4's radiation therapy consisted of 5000cGy divided into 25 fractions of 200 cGy each.

Individual Five was diagnosed at age 38 with invasive ductal adenocarcinoma. She initially had a lumpectomy to remove the tumor tissue, followed by the standard CEF chemotherapy regimen. However, due to her BRCA1 mutation status and advice from her physician, this individual also chose to undergo a bilateral mastectomy and hysterectomy. This is usually considered to be a radical treatment plan; however, this course of treatment dramatically reduces the risk of subsequent carcinogenesis in BRCA1 mutation carriers.

5.5 Discussion of results using study participants

There are two main types of assays done in this study, viability assessments and DNA damage detection. The viability assay that generated significant results was the MTT assay, which will be discussed first, followed by a discussion of the results obtained with the DNA damage assays. Trends or correlations between the cell viability and DNA damage will be highlighted. Lastly, the impact of the BRCA1 mutation on the individual results and the effects of SFN will be discussed.

The MTT assay is a reliable, standard technique which indicates levels of cell proliferation. Absence of proliferation is interpreted as cell death, or loss of viability. From the combined MTT results for all individuals, Individual 1 and 2 have similar responses (Figure 36). Ionizing radiation caused a decrease in proliferation, and when cells were pretreated with SFN there was a cumulative effect, resulting in an additional decrease in proliferation. Individual 3, who is also considered a control, as she does not carry the BRCA1 germline mutation described in her family, had a large increase in proliferation with the SFN pretreatment only, but then a large decrease when treated with ionizing radiation and then a cumulative decrease with both SFN and radiation. However, the experimental error associated with these results indicates that they were not statistically significant. Individuals 4 and 5 had a dramatic response when compared to Individuals 1 and 2. In both individuals 4 and 5, exposure to ionizing radiation significantly decreased cell viability. For both of these individuals, the addition of SFN did not alleviate the effects of ionizing radiation.

From the combined comet assay results (Figure 38) and the γ-H2A.X flow cytometry analysis (Figure 39), Individuals 4 and 5 show an increase in DNA damage in response to ionizing radiation. The results obtained using cells from Individual 3 again show a considerable amount of experimental error, which makes it difficult to interpret the results of experiments using this individual. Interestingly, Individual 5 had the largest increase in γ-H2A.X positive cells following radiation exposure. Individuals 1 and 2 also had significant DNA damage responses, in terms of γ-H2A.X detection. The addition of SFN prior to radiation exposure did not impact levels of DNA damage according to the comet assay. However, there are limitations to the comet assay. The manual scoring system used in this study is highly subjective, even though measures were taken to ensure maximum objectivity, such as blind scoring.

The decrease in cell viability correlates positively with the increase in DNA damage seen following radiation exposure for all the individuals. Cells obtained from Individuals 1 and 2 (the controls) showed a decrease in viability upon exposure to ionizing radiation, as well as a correlating increase in DNA damage. This could be from the large amount of DNA damage that was induced through ionizing radiation that is overwhelming DNA repair mechanisms, and resulting in cell death (Lu et al. 2006). This result is also evident in Individuals 4 and 5 (BRCA1 mutation carriers), as they had the most significant decrease in cell death seen in the MTT assay, as well as significant increases in DNA damage. Based on the results of the MTT assay, ionizing radiation does appear to induce a significant decrease in cell viability in lymphocytes obtained from the individuals who carry a BRCA1 mutation compared to control lymphocytes derived from individuals without a mutation (Figure 36).

In general, SFN had little effect on both the cell viability and DNA damage when administered to the cells 24 hours prior to ionizing radiation exposure. However, the comet assay for Individual 1 showed a reduction in general DNA damage upon the addition of SFN prior to radiation exposure (Figure 38). SFN is a large planar molecule containing a thiol group that is thought to have potent antioxidant capacity (Nguyen et al. 2003). SYTOX Green results indicate that cell death, as measured by loss of membrane integrity, is occurring when cells are treated with SFN only (Figure 40). The addition of SFN could be inducing apoptosis (Myzak and Dashwood 2006), therefore the loss of viability seen in the MTT and SYTOX Green assays may be due to cytotoxic effects. Further experiments are necessary in order to determine the mechanism responsible for the cytotoxicity of SFN. The results of this study indicate that SFN does not impact the loss of viability or amount of DNA damage induced by ionizing radiation.

6. Conclusions

This study is novel because to date there have been no studies that correlate BRCA1 activity with cellular response to radiation therapy using primary cells derived from patients. As mentioned throughout the discussion, the HCC 1937 BL cell line, which is heterozygous for a BRCA1 mutation showed different responses to both SFN and ionizing radiation when compared to primary lymphocytes. The HCC 1937 BL cells were able to withstand a higher dose of SFN (10μM) without exhibiting cytotoxicity, as compared to the dose of 0.5μM used when treating lymphocytes. Also, when HCC 1937 BL cells were exposed to 1000cGy of ionizing radiation, there was very little effect on viability, as compared to the lymphocytes which showed deceases in cell viability. Peripheral blood lymphocytes were used from individuals with BRCA1-associated familial breast cancer and normal controls to compare levels of DNA damage and levels of oxidative stress resulting from radiation exposure.

Mutations in tumour suppressor genes, such as BRCA1, predispose individuals to cancer. BRCA1 is classified as a high-penetrance breast cancer gene, meaning individuals carrying this mutation have an 80% chance of developing hereditary breast cancer by the age of 70 (Boulton 2006). Loss of heterozygosity is commonly observed in hereditary breast cancers, and is one of the most common mechanisms through which the remaining allele is inactivated (Staff et al. 2003). This follows Knudson's "two hit hypothesis" which was first developed in the 1971 (Knudson 1971). The first "hit" or mutation refers to the germline mutation, and the second hit refers to a somatic mutation that disrupts the expression or function of the tumour suppressor gene (Tucker and Friedman 2002). There is a high possibility that the second hit is the stochastic loss of the entire wild-type chromosome 17, which affects TP53 as well as BRCA1. Other potential "hits" are specific microdeletions that affect BRCA1, that can at the same time create a

specific lesion in the PTEN tumor suppressor gene (Foulkes 2008). Without proper functioning or expression of BRCA1, and potentially without a number of functional tumor suppressor genes, the maintenance of genome integrity becomes impossible and the development of breast cancer is inevitable (Saal et al. 2008).

Occurrence of the second "hit" could be a result of an individual's diet. A number of epidemiologic studies have found that there is a link between diet and breast cancer, it has even been estimated that 30-40% of all breast cancer could be prevented by lifestyle and dietary measures (Donaldson 2004). The human diet contains natural and chemical carcinogens, as well as anticarcinogens (Dumitrescu and Cotarla 2005). Some of these carcinogenic compounds, such as compounds found in well done meats (heterocyclic aromatic amines, HAAs) generate free radicals that damage DNA (Dumitrescu and Cotarla 2005). A diet high in saturated fats, refined carbohydrates and concentrated sugars leads to other health conditions such as obesity, diabetes and cardiovascular disease, in addition to cancer (Cabre et al. 2008; Donaldson 2004). Studies have shown that obesity also plays a role in the development of breast cancer in post menopausal women, and it is associated with a poor prognosis in premenopausal women (Sauter et al. 2008). Overall, a poor diet contributes to 50% of breast cancer diagnoses in the United States (Anand et al. 2008). A diet high in fruits and vegetables, rich in antioxidants and phytochemicals, folic acid, vitamins, selenium and a balanced ratio of omega-3 and omega-6 fats could possibly prevent cancer (Anand et al. 2008; Donaldson 2004).

The potential protective effects of a dietary antioxidant, SFN, were investigated. Previous studies involving SFN have found that this antioxidant increases the activity of a transcription factor, Nrf-2, which is responsible for activation of the ARE and therefore, phase II enzymes (Thimmulappa et al. 2002). SFN has also been found to induce cell cycle arrest and

apoptosis in human T-cell leukemia cells; and apoptosis in human osteosarcoma cells (Fimognari et al. 2002; Matsui et al. 2006), and to inhibit histone deacetylases (HDACs) in prostate cancer cells (Myzak et al. 2006). HDACs remove acetyl groups from histones, and therefore preventing transcription of various genes. Currently, there are on-going clinical trials investigating HDAC inhibitors as chemotherapeutic agents (trichostatin A) (Dashwood et al. 2006). HDAC inhibitors disrupt the cell cycle and induce apoptosis through up-regulation of genes such as P21 and BAX. Interestingly, cancer cells are selectively more sensitive to HDAC inhibitor activity than non-transformed cells. SFN is a naturally occurring HDAC inhibitor (Dashwood et al. 2006). This study is also novel because to date no studies have been conducted to investigate the potential antioxidant effect of SFN against oxidative stress induced by ionizing radiation.

The major accomplishments of this study were the development of techniques and verification of methods which can now be used to collect data using a much larger sample size. The SFN dose and radiation dose have been determined, and the techniques used in this study, namely the comet assay, γ-H2A.X flow cytometry analysis, the MTT assay and SYTOX Green analysis were found to be sensitive enough to detect changes upon the exposure of cells to radiation. The hypotheses for this study stated that 1) Radiation damage will be greater in individuals with BRCA1 mutations due to lack of DNA repair capacity and 2) The addition of SFN will protect against ionizing radiation induced damage. Using the above mentioned techniques, it was determined that there was a greater decrease in cell viability in lymphocytes derived from individuals carrying a germline BRCA1 mutation upon radiation exposure. This loss of viability was correlated to an increase in DNA damage. However, based on the results obtained in this study using a very limited sample size, SFN did not appear to alleviate radiation induced damage. To more completely investigate the questions of whether a germline mutation

in BRCA1 significantly impacts the cellular radiation response, and whether the dietary antioxidant SFN protects cells from the damaging impact of radiation, a larger sample size is needed in order for the results to be statistically significant. Because of considerable individual variation in cellular response, the measured differences could be very small, and a larger sample size and the use of proper statistical analyses would be necessary.

There are other facets to this study that have yet to be investigated. These include BRCA1 mRNA expression levels and BRCA1 protein expression levels. The involvement of BRCA1 in sporadic breast cancers is poorly understood. Studies have shown that expression of BRCA1 in high-grade sporadic tumours is absent (Wilson et al. 1999). Absent or reduced expression of nuclear BRCA1 is usually associated with a poor prognosis (Rakha et al. 2008) Also, some research has shown that epigenetic modification of BRCA1 contributes to the development of a certain subset of breast cancers. This histological subset includes medullary and mucinous breast cancers, which are usually BRCA1-inherited breast cancers (Esteller et al. 2000). Investigating how primary blood cells from individuals with sporadic breast cancer react to ionizing radiation in terms of antioxidant response mechanisms, DNA damage repair and BRCA1 protein expression could perhaps help to elucidate the role of BRCA1 in sporadic breast cancer.

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Appendix 1

Regional Cancer Care

980 Oliver Road
Thunder Bay, Ontario
Canada P7B 6V4

Telephone: 807-684-7200

www.tbrhsc.net

Informed Consent

Study Title: **Ionizing Radiation-Induced Oxidative Stress and DNA Damage in Breast Cancer: Role of BRCA1.**

Investigators:
Neelam Khaper, Ph.D. – Northern Ontario School of Medicine, (807) 766-7374
Mary Lynn Tassotto, Ph.D. – Regional Cancer Care, (807) 684-7280
Margaret Anthes, MD – Regional Cancer Care, (807) 684-7211

Introduction
This is a research study. Research studies only include individuals who choose to take part. It is important for you to understand the purpose of this research study. This consent form will describe the research study to you and help you decide if you want to participate in this study. If you have any questions that are not answered, please ask the investigator, who will be happy to answer all of your questions.

You are being asked if you would like to take part in this study because you have been diagnosed with breast cancer, because you have undergone BRCA1 gene testing through the Cancer Genetics Program of the Northwestern Ontario Regional Genetics Program, or because you have been identified as an age- and sex-matched control for this study (an individual who has no personal or family history of breast cancer). This research study is a collaboration between Regional Cancer Care, the Northern Ontario School of Medicine, and the Northwestern Ontario Regional Genetics Program.

Why Is This Study Being Done?
Breast cancer is the most frequently diagnosed form of cancer in women, affecting 1 in 9 women in Canada. The breast cancer susceptibility gene, BRCA1, is known to play a role in hereditary breast cancer. Recent studies have shown that BRCA1 is also important in the development of sporadic (or non-hereditary) breast cancer. At the present time, the function of the BRCA1 gene and the mechanism by which it prevents cancer is not fully understood. This study will help researchers to more fully understand the function of the BRCA1 gene.

BRCA1 has many important functions in a cell. One of these functions is to regulate the cell's oxidative stress response. This study will focus on the role of BRCA1 in protecting cells from oxidative stress. Radiation therapy is an effective treatment used to combat cancer. Radiation kills cancer cells by producing oxygen radicals that damage DNA. This study will attempt to correlate the cellular response to radiation with the level of

BRCA1. Another objective of this study is to determine if dietary antioxidants may alter the cellular response to radiation in individuals with reduced or absent BRCA1.

What is Involved?
This study will use lymphocytes (a type of blood cell) to measure the levels of BRCA1 and correlate this with levels of reactive oxygen species following radiation exposure. The lymphocytes will also be used to measure DNA repair following radiation exposure. To determine if antioxidants protect cells from radiation-induced damage, lymphocytes will be treated with antioxidants before exposing them to radiation, and then their oxidative stress response following radiation exposure will be studied.

For this study, the investigators would like to examine the lymphocyte response to radiation from individuals that have BRCA1 gene mutations and from individuals with sporadic breast cancer (no BRCA1 mutations). This study will help researchers to better understand and predict a patient's response to radiation therapy for breast cancer. This study may also help to improve cancer therapy for breast cancer patients.

In order to conduct this research, one small sample of your blood is required. Your research sample may be shared with other research collaborators for additional biochemical studies of BRCA1 function; however, to protect your identity, all personal identifiers will be removed from your sample and it will be coded with a unique sample number. When the research is complete, the cells isolated from your blood sample will be destroyed. Your consent is required to collect a blood sample. Your consent is also required to have the Northwestern Ontario Regional Genetics Program release to the investigator the results of your BRCA1 genetic tests (if you have been tested through this program). In addition, if you have received treatment for breast cancer at Thunder Bay Regional Health Sciences Centre, your consent is required for the investigators to be able to access your medical records to determine your prior radiation exposure.

How Many People Will Take Part?
In order for the results of this study to be scientifically relevant, the investigators would like to enrol as many individuals in this study as possible. The investigators are hopeful that 120 individuals will choose to participate in this study.

Procedure
A blood sample [20ml (4 tsp.)] will be collected from you. Your blood will be drawn by a nurse, or by a laboratory technologist who is qualified to draw blood.

This procedure will take place at the Thunder Bay Regional Health Sciences Centre. Your participation in this study will require only one visit to the Thunder Bay Regional Health Sciences Centre and is limited to the time it takes to draw your blood.

Risks?
You should be aware of the risks and discomforts that may accompany your participation in this research study. Blood drawing may cause a small amount of pain. In addition, a temporary bruise or "black and blue mark" may develop. Rarely, people faint after blood

drawing. Very rarely, the vein in which the needle has been inserted may become inflamed or infected. This can be treated.

Costs
If you choose to participate in this research study, the investigators can not pay you for your participation. However, your parking fees will be paid when you visit the Thunder Bay Regional Health Sciences Centre to participate in this study.

Benefits
Your participation in this study will not provide any direct benefit to you at this time. However, the information gained in this research study may benefit others by helping researchers to better understand the function of the BRCA1 gene, and by helping researchers to develop more effective radiation therapy treatment for breast cancer patients. The results of this research study may help in the treatment of breast cancer patients in the future.

Confidentiality
All of the information learned about individual participants, including all of their pertinent medical records and results of genetic tests (if applicable) will remain strictly confidential. As a participant, you will never be named in any subsequent reporting of the results of this research study. All blood samples will be encoded with a sample number, which will be assigned to the participant. If the results of this study are used for any publication, your identity will not be released. The data obtained from this research will be stored securely in the research laboratory at Regional Cancer Care or at the Northern Ontario School of Medicine. Research data will be stored for a minimum time of 3 years following the completion of this study before being destroyed.

By signing the consent form, you give permission to the investigators to access your health records from Thunder Bay Regional Health Sciences Centre, and the results of your BRCA1 genetic tests from the Northwestern Ontario Regional Genetics Program (if applicable). This information will not be used for any other purpose than as required for this research study.

Freedom to Participate
Your decision to take part in this research study is entirely voluntary. You may choose not to participate in this study. If you choose not to participate, the quality of care that will be provided to you at any time in the future if you are a patient at the Thunder Bay Regional Health Sciences Centre will not be affected in any way.

You are encouraged to ask any questions about the research study that you may have, and all of your questions will be answered.

If you have any questions about this study or any concerns about your participation in this study, please contact one of the investigators for this project.

Information regarding the rights of research subjects may be obtained from the Thunder Bay Regional Health Sciences Centre Research Ethics Team – Mary Jane Kurm (807) 684-6422. This office has no affiliation with the study investigators.

To be completed by the research subject (Please circle Yes or No):

Do you understand that you have been asked to be in a research study? Yes No

Have you read and received a copy of the attached Consent Form? Yes No

Do you understand the benefits and risks involved in taking part in this research study? Yes No

Have you had an opportunity to ask questions and discuss this study? Yes No

Do you understand that signing this consent form gives the investigators permission to access your medical records? Yes No

Do you understand who will have access to the results of your genetic tests (if applicable)? Yes No

Do you understand that you must also sign an "Authorization for Release of Health Information" from the Northwestern Ontario Regional Genetics Program to give the investigator permission to access the results of your genetic tests (if applicable)? Yes No

Signatures:

I agree to take part in this study.

_____ _____

Signature of Participant Date

This study was explained to me by:

_____ _____

Signature Date

I believe that the person signing this form understands what is involved in this study and voluntarily agrees to participate.

_____ _____

Signature of Investigator Date

Appendix 2

Regional Cancer Care

Research Study: **Ionizing radiation induced oxidative stress and DNA damage in breast cancer: Role of BRCA1.**

Participant Questionnaire

Please complete the following questionnaire. If you have any questions regarding the questionnaire, please do not hesitate to ask the principle investigator.

Participant's Name: _____

Address of Participant: _____

_____

_____

Participant phone number: _____

Participant date of birth: day: _____ month: _____ year: _____

Have you ever been diagnosed with cancer? Yes ☐ No ☐

If yes, indicate primary site: _____

Date of diagnosis: _____

If yes, have you had prior chemotherapy? Yes ☐ No ☐

Date of chemotherapy treatment: _____

If yes, have you had prior radiation therapy? Yes ☐ No ☐

Date of radiation therapy treatment: _____

Do you have any first degree relatives (parent, sibling, or child) that have been diagnosed with cancer? If so, please indicate the type of cancer and how that family member is related to you:

Have you undergone testing for changes (mutations) in the BRCA genes through the Northwestern Ontario Regional Genetics Program? Yes ☐ No ☐

If yes, when did you have the genetic testing done? _____

Have you received the results of your genetic tests? Yes ☐ No ☐

Have any other members of your family been tested? Yes ☐ No ☐

This study is expected to be complete by early 2009. Upon completion of this study, would you like to receive a copy of the results if published?

Yes ☐ No ☐

Thank you for your participation.

Appendix 3

NORTHWESTERN ONTARIO REGIONAL GENETICS PROGRAM

Authorization for Release of Health Information

Permission is hereby granted for the release of information **from** the Northwestern Ontario Regional Genetics Program, to:

_____

Name of Physician or Agency

information regarding:

_____ _____

Name of Adult or Child D.O.B.

_____

Address

_____

concerning the following:

_____

_____

_____

_____

Signature

_____

Relationship to Adult or Child

_____ _____

Date Witness

Personal information on this form is collected under the authority of The Health Protection and Promotion Act R.S.O. 1990 and in accordance with the Municipal Freedom of Information and Privacy Act R.S.O. 1990. This information is collected for the purpose of reporting purposes. Questions regarrding the collection of this information should be addressed to the Privacy Officer, Thunder Bay District Health Unit, 999 Balmoral St.., Thunder Bay, ON P7B 6E7. Telephone (807) 625-8818.

G-14 (FROM) 2004/03/12

THUNDER BAY DISTRICT HEALTH UNIT
999 BALMORAL STREET • THUNDER BAY, ON P7B 6E7
PHONE: (807) 625-5924 • TOLL FREE: 1-888-294-6630
FAX: (807) 623-4341 • E-MAIL: GENETICS@TBDHU.COM

Appendix 4

Memo

Date: 09/18/07

To: Ambulatory Care Staff

From: Mary Lynn Tassotto, Ph.D.

RE: **TASSOTTO COLLECTION STUDY**

The individual presenting this memo to you is a participant in my research study. Please collect a blood sample from the individual. I require approximately **20 ml of peripheral blood** for this study.

- Please collect the blood in sterile vials containing K2-EDTA (***4 pink-topped vials, 6 ml each***).
- Each vial should be labeled with the individual's full name.
- Invert each vial several times to prevent clotting.
- **Do not refrigerate the samples.**

The participant has given his/her consent to have the blood drawn as a part of this research study.

Please immediately contact Dr. Mary Lynn Tassotto, at 684-7280, 684-7247 or 684-7244 to have the blood sample transported from Ambulatory Care to the Regional Cancer Care Research Laboratory.

Thanks, MLT

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Created on 2:56 PM7/29/2008

Appendix 5

MOLECULAR GENETICS DIAGNOSTIC LABORATORY
Children's Hospital of Eastern Ontario
401 Smyth Road, Room W3423
Ottawa, Ontario K1H 8L1
Tel: (613) 738-3230
Fax: (613) 738-4822

Date Sample Received: **25 April 2002**
Date of Report: **4 February 2003**
Previous Report:
CHEO Pedigree No.:
Lab#:

Referring Diagnosis: **Familial or Early Onset Breast Cancer**

Referred by: **Dr. J. Allanson / L. Spooner**
Address: **TB&DHU - Genetic Counselling Service, Thunder Bay**

Pt. Health Care No:

	DATE	INITIAL
PHONED		
SENT	05-02	B
FILED		

RESULTS:

Birth Date day/mo/yr	Name	Ped No	Tissue	DNA Result
	Individual 4	.000	blood	g.56490A>G BRCA1 mutation detected

Analysis: *Approximately 5-10% of breast cancer is inherited and mutations in the BRCA1 and BRCA2 genes have been identified as being the cause of a proportion of these inherited cases. DNA results are based on the sequence analysis of exons 2 and 5 of BRCA1. In addition, analysis for other mutations in these genes is performed using the protein truncation test (PTT). For those individuals who have positive PTT results, sequencing is performed to confirm the presence of a genomic mutation. Mutation designation is based on the recommended guidelines published in Human Mutation V.15:17-12 (2000).*

INTERPRETATION:

DNA analysis has shown that has an A to G change at nucleotide 56490 in the genomic sequence of BRCA1 (previous nomenclature = IVS17+3A>G). This mutation is in intron 17 of the BRCA1 gene and likely effects the splicing efficiency of mRNA in this region. This mutation has been reported previously in the Breast Cancer Information Core Database as an unknown variant. However, the evidence that it does effect splicing is as follows:

1) A PTT positive result was obtained for for the 12 to 24 region of BRCA1.

2) Analysis of the splicing site score for this sequence vs the normal sequence has shown that the score is reduced from 68.4 to 65.2. Although this is not a large reduction, there is an alternate splice site approximately 150 nucleotides down stream which has a score of 68.8, which could be used preferentially over the mutated site.

3) To demonstrate whether this was occuring in her cDNA was sequenced using a reverse primer complimentary to the sequence at the alternate splice site. The resulting sequence included exon 17 and the 5 region of intron 17 with the G at the 56490 nucleotide instead of the normal A. This shows that at least some of mRNA has been spliced using the alternate splice site. cDNA from a normal individual showed no readable sequence as expected. If this alternate site is used, the result is the introduction of a stop codon 42 nucleotides from the 3' end of exon 17, which would explain the positive PTT result from

Unfortunately, using the techniques we have available, we are unable to determine what proportion of mRNA is constructed using alternate splice site. If only a small proportion of mRNA is abnormal, the effect of the mutation may not be enough to increase the susceptibililty for breast or ovarian cancer in family. As this mutation destroys a restriction enzyme site, it may be possible to analyze tissue, if available, from other affected members of family.

This analysis is based on our current knowledge of the BRCA1 and BRCA2 genes.

Nancy Carson, Ph.D.,FCCMG
Head, Molecular Genetics Diagnostic Laboratory

Appendix 6

MOLECULAR GENETICS DIAGNOSTIC LABORATORY
Children's Hospital of Eastern Ontario
401 Smyth Road, Room W3423
Ottawa, Ontario K1H 8L1
Tel: (613) 738-3230
Fax: (613) 738-4822

Date Sample Received: **8 May 2003**
Date of Report: **2 July 2003**
Previous Report: **18 June 2003**
CHEO Pedigree No.:
Lab#:

Referring Diagnosis: **Familial or Early Onset Breast Cancer**

Referred by: **Dr. J. Allanson / L. Spooner; J. Schween**
Address: **TB&DHU - Genetic Counselling Service, Thunder Bay**

Pt. Health Care No:

	DATE	INITIAL
PHONED		
SENT	03-07	B
FILED		

RESULTS:

Birth Date day/mo/yr	Name	Ped No.	Tissue	DNA Result
	Individual 5	.004	blood	g.56490A>G BRCA1 mutation detected

Analysis: *Approximately 5-10% of breast cancer is inherited and mutations in the BRCA1 and BRCA2 genes have been identified as being the cause of a proportion of these inherited cases. DNA results are based on the sequence analysis of exons 2 and 5 of BRCA1. In addition, analysis for other mutations in these genes is performed using the protein truncation test (PTT). For those individuals who have positive PTT results, sequencing is performed to confirm the presence of a genomic mutation. Mutation designation is based on the recommended guidelines published in Human Mutation V.15: 7-12 (2000).*

INTERPRETATION:

DNA analysis has shown that has the g.56490A>G BRCA1 mutation previously described in her family. As has developed breast cancer, this result would support this mutation contributing to the development of breast cancer in this family.

This analysis is based on our current knowledge of the BRCA1 and BRCA2 genes.

Nancy Carson, Ph.D.,FCCMG
Head, Molecular Genetics Diagnostic Laboratory

g.56490A>G BRCA1 mutation

National Human Genome Research Institute
Breast Cancer Information Core Database

Accession Number
4454

Proband Tumor Type
-

Exon
I-17

#Chr **A C G T**
- - - - -

NT
5193+3

Reference
-

Genomic NT
-

Contact Person
A. van der Hout (a.h.vander.hout2medgen.azg.nl)

Codon
-

Notes
-

Base Change
A to G

Creation Date
30-Dec-99

AA Change
-

Ethnicity
-

Designation
IVS17+3A>G

Nationality
Dutch

Mutation Type
IVS

Addition Information
no

Mutation Effect
*

Clinically Important
unknown *

* Insufficient data exist at this time to allow assessment of the clinical or functional implications of this sequence change.

Depositor
A. van der Hout

Patient Sample Source
J. Oosterwijk

ID Number
92/1786

Number Reported
-

Detection Method
DGGE