IMPACT OF NICOTINE ON NON-TARGETED RADIATION EFFECTS

By

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Abstract

Ionizing radiation is without a doubt an invaluable tool in diagnostic imaging as well as radiation therapy. With the growing number of medical and occupational exposures, together with challenges against the LNT model, low dose exposures and non-targeted effects have been subject to intensive research. Additionally, with the advances in the field of radiation therapy and longer life expectancy after the treatment, the risks associated with second malignancies following radiation therapy for various cancers has received a tremendous amount of attention. On the other hand, nicotine, as the addictive component of tobacco has been known for its adverse health effects and its relation to various types of cancers, accounting for one in 10 adult deaths worldwide. Both nicotine and low doses of radiation are amongst the stressors that widely affect the public. Surprisingly, the interactions between low-dose effects and nicotine exposure have not received the proper scientific attention. Our group has been involved in investigation of the non-targeted effects of radiation with a variety of endpoints. Different natural compounds and signalling molecules have also been studied in our lab for their possible role or contribution to bystander signalling. This research involves the study of the impact of nicotine on radiation-induced bystander effects and also radioadaptive responses. Different concentrations of nicotine were used to study the kinetics of the drug as well as any detrimental or modifying effects when used together with radiation. It was shown that nicotine has a protective effect on survival of the cells in certain

concentrations that follows a biphasic model. Similar bimodal behaviour was observed with bystander effect. No adaptation to a challenge dose of radiation occurred as a result of incubation with varying concentrations of nicotine, nor was such an effect shown with a priming dose of radiation. The results of the present study suggest that nicotine has a complicated effect on the cells which can vary significantly depending on the concentrations used and also the duration of exposure. nAChRs may have an important role in the response of the bystander cells when nicotine is involved as the results showed a shift in the response of the receptors to nicotine. This thesis is aimed to shed light on the impact of nicotine and initiate more detailed investigations on pathways through which these effects are mediated.

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List of Abbreviations

Ach	Acetylcholine
CCCM	Control Cell Conditioned Medium
EC	Endothelial Cells
ECM	Extra Cellular Matrix
FBS	Foetal Bovine Serum
HPV	Human Papilloma Virus
ICCM	Irradiated Cell Conditioned Medium
ITCM	Irradiated Tissue Conditioned Medium
LET	Linear Energy Transfer
LGIC	Ligand Gated Ion Channels
LNT	Linear No-Threshold
LQ model	Linear Quadratic model
nAChR	Nicotinic Acetylcholine Receptor
NO	Nitric Oxide
ROS	Reactive Oxygen Species

Chapter 1

Background and Motivations

1.1- Nicotine

Nicotine is undoubtedly a drug with tremendous cultural, scientific, economical, industrial, and health impacts on the society [2]. The cultural impact of nicotine dates back to at least 2000 years ago when Native Americans used the drug for the purpose of spiritual rituals and held it sacred. The recovery from death-like states induced by nicotine which was in the form of a coma was considered to be a supernatural power. It was also used as a powerful medicine by Shamans for guidance, pain relief, and healing, believing tobacco was given by immortals to humans to guide them from past to present and future [3].

The economical and industrial implications of nicotine are closely tied to tobacco industry. The addictive properties of nicotine maintain the viability of this profitable industry while the cost of smoking remains to be a considerable economical burden on the society. Nicotine is also used in pesticides due to skin absorbance and also recently in pharmacology for manufacturing new drugs [2].

Scientifically, nicotine has been widely used to probe receptors and also to identify their subtypes. Indeed, identification of cholinergic receptors and their subtypes and assembly has and will continue to have a major contribution in better characterizing different disorders of the central nervous system such as addiction, depression, and a number of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. The recent discovery of non-neuronal nicotinic receptors has also opened the door to exciting areas of research and will be addressed to a greater extent in the following pages of the present research [1, 3].

The health effects of nicotine are those associated with smoking cigarettes, as nicotine itself in most cases is not the cause of smoking-related diseases. However, being the addictive ingredient in cigarettes, it contributes to ongoing use of cigarettes and consequently exposure to a wide range of carcinogens. Therefore, nicotine is not classified as a carcinogen, but there is debate among researchers whether it should be considered as a tumor enhancer. Most of the diseases associated with smoking, and thus nicotine, are well known and include but are not limited to addiction, a number of cancers, such as lung cancer, cancer of the oral cavity, malignant melanoma [4], cardiovascular diseases, and also premature deaths as a result of smoking during pregnancy [2]. There are also tissue and organ specific diseases and complications, a few of which are aging and inflammation [5]. A 2009 report of the world health organization states: *'currently, tobacco use kills 5.4 million people per year- an average of one person every six seconds- and accounts for one in 10 adult deaths worldwide'* [6]. Smoking

alone accounts for 90% of lung cancers which is the leading cancer-related cause of death in the world [4]. Another rare health effect associated with nicotine in poisoning as a result of accidental exposure to pesticides. The regulations in effect to limit public health risks of nicotine are enforced in many countries and include awareness about the detrimental health effects and high taxation [2].

Nicotine is one of the few naturally occurring alkaloids and can be found predominantly in genus Nicotiana plants. They can also be found in variety of other plants, such as horsetail, cauliflower, eggplants, tomatoes, and potatoes [7]; the amount of nicotine in which is small enough not to cause any physiologic effects, but may be traceable in urinary cotinine levels of non-smokers [8].

1.1.1- Drug Form and Relevant Numbers

In any experiment involving nicotine, it is important to choose the doses that are relevant to human use of tobacco in any form, or the concentrations used in certain medications. As far as the lab experiments are concerned, nicotine can be obtained in two major forms. First is the free base which is in liquid form with molecular weight of 162 g/mol. The other which comes in several different forms is nicotine tartrate with molecular weight of 462 g/mol. investigators of this field strongly suggest the use of nicotine in its free base form for all studies [9].

Cigarette tobacco contains on average 1-2% nicotine which translates into 0.8-1.9 mg of nicotine per cigarette. Taking into account the average body weight of 150 lb (1 kg= 2.2 lb), an average cigarette delivers about 10-30 μg/kg. Dividing this value by the molecular weight of nicotine yields the concentration in molarity. Therefore, the typical nicotine concentration in blood plasma using the above numbers is about 0.31 μM. It has been shown in experimental animals that the nicotine levels in breast milk after chronic exposure is almost 3 times the plasma levels [9].

1.1.2- Uptake and Distribution

Nicotine is normally consumed through cigarette smoking, oral snuff, pipe tobacco, cigars, and chewing tobacco; the first three contain the same amount of nicotine, where as cigars and chewing tobacco deliver half the concentration of cigarettes [10]. Based on an American study, a smoker consumes 17 cigarettes per day on average [11], resulting in evening plasma levels of 0.06-0.31 μM and minimum concentration ranges of 0.03 to 0.23 μM [12]. The variation in concentration values is the consequence of the complex process of smoking, varying for each individual on puff to puff basis [10].

Once inhaled, nicotine is absorbed through the alveoli in the lungs and from there to arterial blood stream through which it reaches the brain in 8-10 seconds and results in the psychoactive effects of the drug [9]. About 20 minutes after smoking the blood levels drop dramatically, this is the result of the distribution of nicotine throughout the body organs [9]. Nicotine has a high affinity for the liver, kidney, brain, spleen, and lungs.

Lowest concentrations can be found in the adipose tissue [13]. Nicotine also crosses the placental barrier; studies have shown that nicotine levels in amnionic fluid are even slightly higher than the maternal serum [10].

1.1.3- Metabolism

Nicotine is metabolized by the liver to a number of metabolites, six of which are considered to be important metabolites (Fig. 1) [10]. The major metabolite is cotinine constituting more than 70% of the primary metabolism. Cotinine is often used as a marker for urine analysis in tobacco addiction tests, as only 5% of nicotine gets excreted by the kidney unmetabolized. Many animal species, including mice, dogs, and rabbit metabolize nicotine in the same way as the humans do; exceptions are rats and guinea pigs which renders them unsuitable as an animal model in nicotine related studies. Nicotine has a plasma elimination half life of about 2 hours; however, nicotine levels of about 0.03 μM are still present in a smokers blood in the morning, assuming overnight abstention [9]. The rate of nicotine metabolism determined through blood level measurements has an average of 1,200 ml/min which is slower after chronic exposures. Non-renal clearance represents about 70% of the blood in the liver. In other words, in each passage of blood through the liver 70% of nicotine is cleared from it [4, 3]. Many different factors are in effect when it comes to nicotine metabolism in humans. Some of these factors include age, sex, diseases and also race. Elderly people metabolize nicotine

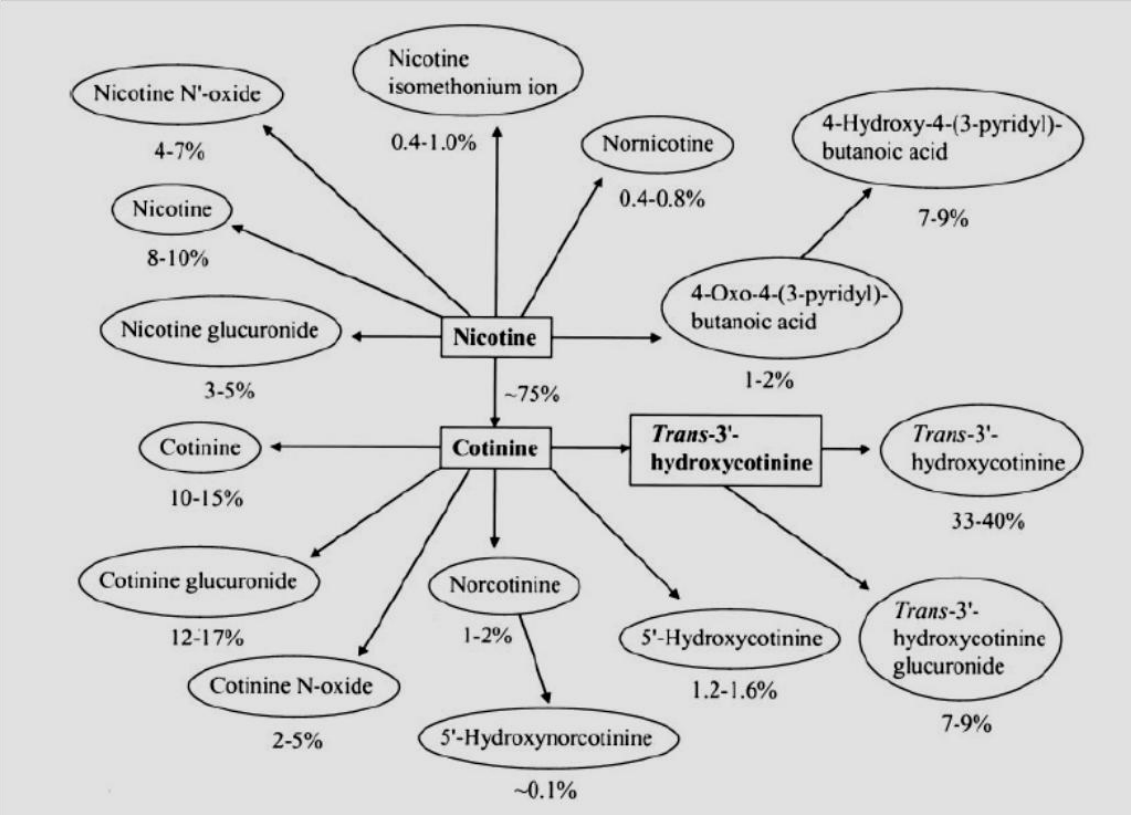


Figure1.1- Quantitative scheme of nicotine metabolism, based on urinary nicotine data [1]

23% slower when compared to younger adult smokers [14], which could be due to slower blood flow and enzymatic changes in liver, as well as slower kidney function [9]. Women metabolize both nicotine and its major metabolite cotinine faster by 13 and 26%, respectively [9]. The same holds true for pregnant women with the rate rising up to 60% and 140% [15]. Asians metabolize nicotine more slowly than Caucasians while African Americans metabolize it faster and Latinos at the same rate [10, 11].

1.2- Nicotinic Acetylcholine Receptors

In 1921 Otto Loewi and Henry Dale identified acetylcholine (Ach) as a fundamental neurotransmitter. They were awarded the 1936 Nobel prize for physiology and medicine. This discovery was followed over the years by characterizing its two receptors, nicotinic and muscarinic [18].

The adverse effects of nicotine are mediated through nicotinic acetylcholine receptors (nAChRs), the function and expression of which has a fundamental role in nicotine addiction. The process of nicotine addiction and tolerance is a complicated one, involving many different pathways and transmitters; however, nicotinic receptors are the main mediators of the effects downstream from the intake of nicotine.

Until about a decade ago it was assumed that nAChRs were only expressed in the brain and ganglionic receptors of the peripheral nervous system. Recently, various subunits of the nicotinic receptors have been identified in different tissues and organs; some examples are bronchial epithelial cells, keratinocytes, and arterial endothelium, as well as cancer cells [19, 20].

nAChR are acetylcholine receptors belonging to ligand-gated ion channel (LGIC) family which have a wide distribution throughout the brain and the rest of the human body [21]. The shape of the receptor represents an assembly of 5 subunits arranged symmetrically around a central pore [22]. There are two main groups of subunits, namely α and β. So far 10 α (α1-α10) and 4 β (β1-β4) subunits have been identified which

support a wide variety of subunit arrangements, rendering different functions depending on the location of the receptor. The permeability of the receptors depends upon the composition of the subunits; in general, nicotinic receptors are permeable to K^+, Na^+, and also Ca^{2+} ions [6]. Involvement of calcium channels in radiation effects and especially bystander signalling are already shown and accepted [23]. α subunits have the ability to bind to agonists; α7 nAChR is one the most abundant subunits and can be activated even with low concentrations of agonist [24]. As the concentration of agonist increases, so do the occupied binding sites, resulting in a rapid, but short-lived desensitization of the receptor [24,25]. Nicotine is an agonist of acetylcholine; the binding of either Ach or its agonists opens the pore of the receptor, allowing the influx of the cations into the cell, thus changing the electrical equilibrium and either initiating or inhibiting an action potential [24,26]. The main difference, however, lies in the degradation process of acetylcholine and nicotine; while acetylcholine is degraded and removed rapidly, nicotine remains in the vicinity of the receptors for longer periods of time. The average residence time of Ach is less than 1ms [27], while nicotine is metabolized through liver enzymes with a half life of 120 minutes [9,24]. Therefore, the effect of nicotine is mediated through desensitization rather than activation [24]. Chronic exposure to nicotine causes the upregulation of nicotine binding sites, both in neuronal and non-neuronal receptors; this upregulation is in fact an increase in the number of nAChR which in turn translates into increased sensitivity to nicotine. The pathways to the upregulation in response to nicotine, however, are poorly understood [28]. Considering the wide range of functions

attributed to nAChRs, it is possible that they also play a role in mediating the radiation and bystander effects, which will be investigated further in this thesis.

Biological effects of nicotine are generally categorized into effects on neuronal cells and non-neuronal cells, as the cell lines used in the present study are of non-neuronal origin, the emphasis of the effects will be on those related to non-neuronal cells.

1.3- Nicotine-induced Effects on Non-neuronal Cells

Since the discovery of non-neuronal nAChR, many researchers have focused their studies on characterizing and understanding the effects of nicotine on different cells and tissues. The initial studies were mostly done on lung cancer cell lines which showed that nicotine in fact induced the release of serotonin [28,29]. Interestingly, serotonin is also involved in bystander signalling [30]. To this day, most studies have been essentially focused on three different effects; these are angiogenesis, proliferation, and apoptosis [6,10].

1.3.1- Neo-angiogenesis

The result of a study published in 2001 showing antigenic properties of nicotine triggered scientists to study the possible correlation between nicotine-induced angiogenesis and diseases such as cancer and cardiovascular diseases which are closely related to tobacco use. Angiogenesis is mediated through nAChRs on endothelial cells

(EC) which may be of physiological or pathological nature; once the balance between the growth factors is lost in favour of angiogenesis, the endothelial cells in the existing vessels are stimulated to proliferate and migrate to form new blood vessels, hence the term neo-angiogenesis [6]. Interesting *in vitro* research in this field suggests a bimodal response where nicotine concentrations of below 10^{-8} M in the blood induce EC proliferation and those above 10^{-6} M cause cytotoxicity [31]. Other studies also support such behaviour which varies depending on concentration and duration of exposure. α7 nAChR subunit has shown to have a pivotal role in neo-angiogenesis. The same effects with respect to angiogenesis and tumour growth have been reported in various tumour cells, which were inhibited once nAChR antagonists were introduced [6,10].

1.3.2- Proliferation

Nicotine-induced cell proliferation has important implications both in normal and cancerous tissues and cells, therefore, a large number of studies have focused on these effects in various cell lines, some of which are briefly mentioned in this section.

Generally, α7 subunit has been associated with cell proliferation in many tissues and cell lines, as α7 nAChR antagonists can attenuate and inhibit the proliferative effects of nicotine. Exposure to nicotine in human keratinocytes causes changes in mRNA protein levels of different markers these cells, such as those involved in cell cycle and differentiation; examples are p53 and cyclin D1 [10,32]. The pathways through which nicotine induces its proliferative effects include increasing both the growth factors and

also the growth factor receptors. The latter is induced by increasing intracellular levels of Ca^{2+}, this is particularly important in signalling pathways downstream of α7 subunit activation [33]. Chronic exposure to nicotine can alter the composition of subunits in favour of α7; this was shown in human keratinocytes where the composition change observed was α7 in place of α3 nAChR. Other subunit composition changes have also been observed in these cells [32].

Human bladder cells synthesize and secrete Ach; nAChRs are also found in these cells with a gradient of intensity in their expression depending on the type of cells. In human bladder cancer cell lines, a concentration of 1 μM induced resistance to chemotherapy treatment by upsetting the regulation of the cell cycle through upregulation of cyclin D1. The results of similar studies on other tissues and cells support the proliferation induced by nicotine [6].

1.3.3- Apoptosis

Apoptosis, or programmed cell death, not only happens during development and aging as a normal mechanism to maintain tissue homeostasis, but also acts as a response to external damaging stimuli, such as drugs and radiation [34]. Nicotine, like radiation, has an influence on apoptosis and has been studied both *in vitro* and *in vivo* in many different tissues and cell line by various research groups [21].

The first evidence of involvement of nicotine in apoptotic mechanisms was observed before the presence of non-neuronal nAChRs was accepted, where chronic exposure to nicotine weakened the effect of anticancer drugs in leukemia cells [6]. Research results support both anti-apoptotic and pro-apoptotic effects depending on the cell line studied, concentrations of the drug, and *in vivo* versus *in vitro* investigations. As far as the *in vitro* studies are concerned, usually primary mammalian or permanent cancer cell lines are used; even though the generally accepted mode of action is through nAChRs, very few studies actually investigate the presence or status of these receptors in their model system. A study done by Wright showed that nicotine concentrations of 10 μM, 100 μM, and 1mM inhibited apoptosis induced by several chemotherapeutic drugs, which lead the authors to conclude that nicotine may cause new tumors and reduce the efficacy of cancer treatment [35]. Mai and co-workers also reached the same results after treating lung cancer cells with 1 μM of nicotine, observing that a specific phosphorylation of bcl-2 led to a higher survival rate [36]. Heusch and Maneckjee's work, while confirming the above results, showed that activation of mitogen-activated protein (MAP) kinase signalling pathway after nicotine treatment increased the expression of bcl-2, thus inhibiting apoptosis [37]. Nicotine also increased the number of cultured human umbilical vein endothelial cells by three fold with nicotine concentrations as low as 10-100 nM, as shown in the results of Heeschen's group. In addition, the number of hypoxia-induced apoptotic cells was decreased.

There are also a few studies that support the pro-apoptotic effects of nicotine. These pro-apoptotic effects, however, are weak when compared to the results of anti-apoptotic studies [21]. One pathway most commonly observed is a significant increase in caspase 3 activity which results in apoptosis [21,38]. A bimodal response was also described in calf pulmonary endothelial cells where concentrations of nicotine as low as 0.1 nM-10 nM stimulated proliferation, while higher concentrations caused a diminished DNA synthesis and apoptosis [31].

To summarize, only a minority of in vitro studies support the pro-apoptotic activities of nicotine, and most of these studies report small effects. Given the heterogeneity of the experimental settings, cell lines used, endpoints, and techniques employed, it is difficult to compare the results and make a solid conclusion about the effects of nicotine.

1.4- Radiation Damage

Radiation is with no doubt an invaluable tool in diagnostics and treatment with various clinical applications. Different fates may await a cell after being traversed by radiation tracks. If the damage is significant, the cell loses its functionality, leading to cell death [39]. The second possibility is the loss of reproductive ability and the third is erroneous repair of damage, leading to impaired future copies of the DNA [39–41].

Cell death can be induced in different forms; the primary mode of cell death after radiation-induced injury in some cells is apoptosis or programmed cell death, characterized by membrane blebbing, cell shrinkage, and chromatin condensation. On a molecular level, mitochondrial membrane depolarization and rupturing of the plasma membrane are the most prominent characterizations of apoptosis [42,43]. Apoptosis is known as a crucial component of various cellular and physiological functions including normal cell turnover, development of the immune system, embryonic development and chemical-induced cell death [34]. Another mode of cell death, senescence, is characterized by failure to duplicate the DNA while the cell remains active metabolically [44]. Mitotic cell death, another common form of cell death is associated with a failed attempt to complete mitosis. Cells experiencing mitotic death may make it through a couple of mitoses before cell death occurs. Terminal differentiation happens when a cell ceases to divide permanently. It differs from apoptosis in that no membrane blebbing is observed and the process takes much longer (days as opposed to hours) to complete [44].

1.5- Targeted Effects of Radiation

The publication of two books on actions of ionizing radiation on living systems started the era of radiobiology. These were “Actions of radiations on living cells” in 1947 by Lea and “Das Trefferprinzip in der Biologie” by Timofeeff-ressovsky and Zimmer in 1947 [45]. There are three historical approaches that attempted to explain the observed effects at the time. The first was Lea’s “Target Theory”. Lea’s model was specific to low

dose radiation and assumed that a cell can have one or more targets that can be hit by one or more radiation tracks. The case that gained applicability in radiobiology was the "multitarget-single-hit" version. According to this model there are multiple targets in one cell with equal probability of being hit. Each hit would be enough to kill a target but not the whole cell. Having also a single-target single-hit component, this model supported the response of most mammalian systems. The important shortcoming of Lea's model was that it assumed a constant slope with increasing dose in the linear part of the survival plot, whereas the experimental data supported an increasing slope [45,46]. DNA has outstanding damage repair capabilities which Lea's model did not take into consideration. This led to the introduction of an alternative model. In their publication of 1973, Chadwick and Leenhouts explained the theory by the name of "molecular model", more widely known as the "linear quadratic model" (LQ model). Based on this model, double strand breaks (DSB) in the DNA helix was the ultimate damage and took into consideration various damage repair mechanisms. The extent of the repair of the breaks in molecular bonds of the DNA caused by radiation resulted in different radiobiological outcomes [46,47]. Even though this model successfully overcame the issues of the previous model and is in use in radiobiology, it still suffered inconsistencies with the experimental data over the assumption of a proportional correlation between DSBs and induction of lethal lesions [46]. An upgrade to the LQ model was proposed by Kellerer and Rossi in 1973 [46]. This model was the result of an attempt to explain the increased neutron RBE at low doses and also the results of microdosimetry studies done by the authors. This model was based on two assumptions; firstly, radiation causes dose-

dependent sublesions and secondly the interaction between sublesion within a specific distance may lead to a lesion. This model, however, received criticism by many research groups due to discrepancies with the LQ model [46].

During the past decade a plethora of published research that support non-DNA targets have been increasingly questioning the validity of the LNT model. The argument is mainly over extrapolation of risk estimates from high doses for which an actual human data exists to low doses, as different mechanisms are in effect at low doses comparing to high doses. This is discussed in more detail in the following section.

1.6- Non-targeted Effects of Radiation

In recent years the standard model for radiation effects has been challenged by a large number of researches showing various cellular responses to ionizing radiation which occur in the absence of direct DNA damage. These effects are referred to as non-targeted effects of radiation and have drawn great interest among physicists and biologists during the last 50 years leading to a paradigm shift in the field of low-dose effects. Kuhn defined 'paradigm shift' as '*an intellectually violent revolution in which one conceptual world view is replaced by another, and non-targeted effects very well fit to this definition'* [48]. The "linear no-threshold model" (LNT model) has been widely challenged by these effects, however, whether it leads to an overestimation or under estimation of cancer risks is subject to debate and may vary depending on the dominant effect. These effects include bystander responses, adaptive responses, low dose

hypersensitivity, inverse dose-rate effect, gene expression, and genomic instability [49]. The last four will be only briefly mentioned as they were not investigated in this research, while the first two will be discussed in more detail.

1.6.1- Low Dose Hypersensitivity

Low dose hypersensitivity can be defined as excessive cell death in response to extremely low doses (0.1 Gy) of radiation which deviates from the low dose response predicted by standard LQ model. This behaviour usually continues until 0.3 Gy, after which is followed by radioresistance up to 1 Gy. Thereafter, the response follows the standard dose-dependent behaviour. This phenomenon has been observed in many mammalian and non-mammalian cell lines following exposure to both High and low LET radiations [49–51].

1.6.2- Inverse Dose-rate Effect

Generally, biological effects of radiation weaken as the dose-rate decreases. However it has been shown that at very low dose-rates (0.1-1 cGy/min) there exists an inverse dose-rate effect where more mutations are observed. This is referred to as the inverse dose-rate effect of radiation and has been observed in both somatic and germ-line cells [49,52].

1.6.3- Genomic Instability

Genomic instability is known to be a key step in cancer which may be inherited or induced. It is defined as genome-wide changes which occur in the progeny of irradiated cells. These changes may comprise chromosomal changes, mutations, and delayed cell death. Genomic instability is one of the well-studied endpoints in radiation research and seems to be dependent on cell line, its genetic background, and radiation type [53].

1.6.4- Gene Expression

Several studies have reported upregulation or down regulation of some genes in the dose ranges that induce no significant level of damage to cells. Doses as low as 2 cGy, at which no apoptosis or decreased cloning efficiency can be detected, are shown to cause changes in certain genes, which suggest that DNA damage is not necessary in induced changes in gene expressions [54].

1.6.5- Bystander Effects

Bystander effects are referred to the damages and effects induced in cells that have not been traversed by radiation tracks but are in the vicinity of the directly exposed cells [49,55–59]. The phenomenon was first reported by Nagasawa and Little in 1992 where they observed sister chromatid exchange in 20-40% of the cells whereas only 0.1-1% of the cells were actually hit by α-particle tracks [56,60]. These findings were

confirmed through the use of different endpoints by different research teams [61]. Using lung epithelial cells from rats, Hickman *et al* observed increased TP53 protein levels which were higher than what was estimated based on the number of cells hit by alpha radiation [62]. All of the aforementioned studies used α-particles as the source of radiation. An increase in the number of sister chromatid exchange was also reported in the work of Smith *et al* using x-rays, confirming that the effects were not limited to high LET radiation [63].

Two experimental approaches have been employed to study the bystander effects. First is through irradiation of monolayer of cells with low fluence of alpha particles in a manner that only a small fraction of cells in the population are hit by the radiation tracks [56,64–66]. An alternative to this form of irradiation is using sophisticated microbeams to precisely irradiate single cells which is limited to a few laboratories in the world [67,68]. The second approach uses the cell-conditioned medium harvested from cells exposed to low LET radiation (i.e. x-rays and gamma rays) after a suitable incubation time and replaces it with medium from the unirradiated cells. The current research employs this method of investigation for assessing the impact of nicotine on radiation-induced bystander effects [30,69–72].

Mothersill and Seymour performed extensive studies involving medium transfer from irradiated cells. They established the bystander effect following low-LET gamma radiation *in vitro*, by filtering the supernatants from the irradiated cells and exposing it to unirradiated cells using clonogenic survival as their endpoint. They observed a significant

decrease in survival. No toxic effect was seen when cells were exposed to irradiated media with no cells. This excluded the possibility of radiation having an effect on the culture medium [73]. In a separate study they exposed the recipient cells to irradiated cell conditioned media (ICCM) for various lengths of time and observed a saturated effect after 30 minutes of exposure. This observation led them to conclude that detrimental effects on cell survival was the results of a signalling cascade rather than cytotoxic factors released in the media [55]. In an effort to show the effect in different cell lines, Mothersill and Seymour observed that unlike epithelial cells, fibroblasts were incapable of producing a cytotoxic signal, nor were they able to receive the signal generated by capable cells [73]. This study was extended to different cell lines and also tissue samples from various irradiated fish, which confirmed the cell line dependent nature of the signal [74–76]. In subsequent experiments, their group showed that the diminished survival was correlated to cell density of the donor flasks and at the same time independent of the cell-cell communication. This was concluded based on the observation that the effect was not diminished after administration of an inhibitor of gap junction communication (phorbol myristate acid) [77].

1.6.5.1- Mechanisms of Radiation-induced Bystander Effects

The mechanisms underlying the radiation-induced bystander effects are not fully understood and are subject to ongoing debate. However, signal transduction between irradiated and unirradiated cells plays a major role. The research in this area is

categorized based on whether the irradiated and unirradiated cells are neighbour cells or not. Signal transmission through intercellular junction communication or through interactions between ligands and their specific receptors are the methods that require the two groups of cells to be in contact, i.e. neighbours. The former has been studied by many groups [64,65,78,79]. Azzam et al irradiated human fibroblast cells with 0.3 cGy α-particles where 2% of the cells where hit by radiation and observed a high induction of TP3/CDKN1A signalling pathway which was reduced by administration of lindane, a gap junction inhibitor, thus proving that the effects observed were due to gap junction communication between the irradiated and unirradiated cells [64]. Effect on non-uniform distribution of radioactivity among cells using tritiated thymidine labelling, was assessed via cell survival assay comparing 100% labelling and 50% labelling. Interestingly, the effects observed after 50% labelling could be reversed by lindane, whereas lindane showed no effect on the 100% labelled group, elucidating the role of bystander effect in the survival of V29 cells via gap junction communication [78]. The interaction between ligands and receptors was shown in the work of Albanese and Dainiak. They reported a dose-dependent upregulation of TNFSF6 in a colon cancer cell line induced by ionizing radiation. TNFSF6 is known as "death" ligand and belongs to the family of plasma membrane-bound growth regulators. Western blot analysis of the vesicles showed a high level of TNFSF6 after a dose of 10 Gy compared to that of the controls [80].

Signal transmission between irradiated and non-irradiated cells through interaction between secreted factors and specific receptors or directly through plasma

membranes are the two possible mechanisms which are based on the non-adjacent cell approach [60]. Mothersill and Seymour demonstrated a reduction in survival of normal human keratinocytes after exposure to irradiated cell-conditioned medium in low doses. The fact that the response was a temperature dependant one, sensitive to both high and low temperatures (0< and >70 °C), led them to suggest that the secreted factors could be proteins [55]. Iyer and Lehnert's work on secreted factors employed exposure of unirradiated lung cancer cells to supernatants of α-irradiated cells. They reported an increase in the growth factor TGF-β1 in the supernatants which subsequently caused the intracellular reactive oxygen species (ROS) to increase and TP53 and CDKN1A to decrease. The authors concluded that increase in intracellular ROS is a key step in mediating the bystander effects following high LET radiation [66]. Narayanan *et al* also reported intracellular generation of superoxide and hydrogen peroxide in human lung fibroblasts exposed to the serum of α-irradiated cells, further confirming a role of ROS in radiation-induced bystander effects [81]. Direct signalling through plasma membrane was shown through the continuous work of Matsumoto *et al*. They observed an accumulation of TP53 and showed the importance of the signalling pathway initiated by nitric oxide (NO) in response to bystander signal in human glioblastoma cell lines [60,82,83]. Calcium influx following exposure to ICCM seems to play an important role in the bystander signalling pathways. Many studies have confirmed a rapid spike of calcium measured immediately after addition of ICCM to the reporter cells suggesting that this influx may be one of the initial steps in the signalling cascade [23,71,84,85].

1.6.5.2- Radiation-induced Bystander Effects in the Context of Multiple Stressors

Different implications of radiation induced bystander effects with respect to environmental issues, public health, and patient care have driven the attention of some research groups. Mothersill and Seymour have done extensive research in this area and studied many environmental and natural chemicals. The present research also falls into this category of research, studying the effects of nicotine on low dose radiation, radiation-induced bystander effects and adaptive responses. Influence of melanin on RIBE as a natural substance with potential radioprotective properties was tested by Mosse *et al*. Melanin is more effective in lower dose ranges. Their results showed that melanin enhanced the colony forming ability of the bystander cells when melanin was added prior to irradiation, even though there was no melanin in the filtered medium as proven by absorption spectrum. Adding melanin after irradiation offered less protection in both directly irradiated and bystander cells. The authors concluded that bystander effect may have a physical component based on the fact that melanin is capable of absorbing all types of energy [72]. Poon *et al* tested small signalling molecules and also drugs such as serotonin, l-DOPA, glycine, and nicotine to investigate the hypothesis that these molecules may have an influence on RIBE. Serotonin was depleted following irradiation, an indicator of possible binding of the neurotransmitter to the membrane receptors. This effect was blocked by administration of inhibitors of serotonin and also reserpine, an antagonist of the serotonin. Nicotine in nanomolar ranges enhanced survival and glycine

also enhanced growth comparing to directly irradiated cells in the nanomolar and micoromolar concentrations [30]. In 2007 Mothersill *et al* investigated the environmental impact of multiple stressors. They investigated the impact of combination of aluminum, cadmium and low-dose radiation in Atlantic salmon fish. The fish were exposed to 0.5 Gy of gammas in water that contained Al or Cd or both in subtoxic concentrations. Relevant organs were collected and the irradiated tissue conditioned medium (ITCM) induced a bystander effect in all cases. The effects however were not consistent and a significant variation between different organs for different treatments was observed. An important observation of this study was the tissue-specific nature of the effect [86,87].

1.6.6- Adaptive Response

Radioadaptive response is described as reduced detrimental effects of high doses of irradiation when induced after a low dose priming exposure [50]. Adaptive responses were first reported by Olivieri *et al* in 1984. Human lymphocytes labeled with tritiated thymidine were exposed to 1.5 Gy X-rays at 5, 7, 9, and 11 hours prior to fixation. The number of chromatid aberrations was less than the sum of chromatid aberrations in radiolabeled thymidine or X-rays alone [88]. Since then many studies have reported and reviewed the adaptive responses *in vivo* and *in vitro* using different endpoints. The results have been subject to tremendous variability which may be attributed to dose, time between doses, as well as genetic variability among different individuals [50]. Zhou *et al* (2003) investigated the interaction between adaptive responses and bystander effect by

exposing the cells to low doses of X-rays 4 hours prior to α-particle irradiation using microbeam technology. They reported a decrease in the bystander mutagenic response and also an elevated sensitivity in the bystander cells which received a challenge dose of X-rays [89]. There have also been studies showing the absence of an adaptive response. Wojcik *et al* reported no decrease in the number of chromosomal aberrations when isolated mouse lymphocytes were exposed to 0.1 Gy followed by a challenge dose of 1.5 Gy after 48 hours [90]. In 2002 Sorensen *et al* tested 10 different human lymphocyte cell lines and reported varying results ranging from no adaptive response to adaptive and also synergistic effects [91]. A wide range of dose and dose-rates have been studied in different cell cultures. In general, adaptive responses were observed in 0.01-0.5 Gy and 0.01-1 Gy/min as priming doses and dose-rates, respectively [50]. The time interval between the administration of the priming dose and the challenge dose has a high impact on the effect observed and also its magnitude as shown by different groups. The range varies from a few hours up to 40 days. Maguire *et al* showed a significant effect when the challenge dose was administered 24 hours post the conditioning dose [92]. Cai *et al* exposed mice to a chronic low dose-rate of X-rays for 40 days and challenged the mice 40 days after with a subsequent large dose of X-rays and saw reduced cytogenetic effects [93].

Animal studies of Radioadaptive responses have been conducted by many research groups as shown above. Ryan *et al* showed a ‘protective’ bystander response in three different fish cell lines that were given a 0.1 Gy conditioning dose eight hours

before the challenge dose. The ICCM derived from the primed fish cells caused an increase in cloning efficiency in unirradiated reporters, when compared to the controls [94]. Moskalev *et al* in their published work of 2011 used Drosophila Melanogaster flies as their model system and showed that chronic exposure to doses as low as 40 cGy induced a hormetic effect that protected the flies from the subsequent challenge dose of 30 Gy. They also observed strain-specific and also gender-specific differences in their results [95]. Mitchel *et al* examined radiosensitive and cancer prone mice (heterozygous for Trp53) with a high probability of spontaneous cancers. They exposed the mice to 10 mGy and 100 mGy of Co-60 gammas at a low dose-rate of 0.5 mGy/min. Interestingly, they observed that the exposure had no effect on the spontaneous rate of cancer; however, both 10 mGy and 100 mGy increased the latency period of the cancers, with 100 mGy showing a more significant increase. They concluded that this low dose-rate exposure slows down the malignancy progress in these mice [96].

1.6.6.1- Environmental and Occupational Implications of Adaptive Responses

Just like bystander and other non-targeted effects, adaptive responses also challenge the validity of LNT model in the low range region. Implications of adaptive responses has been shown by different groups, however, the focus has mostly been on areas with high background radiation levels. Such areas are found in Brazil, China, India, and Iran [50]. Ramsar in Iran has a background level 5 times higher than normal

background. In a study done in 2002 by Ghiassi-nejad *et al*, lymphocytes from local people as well as those of the inhabitants of normal background areas were exposed to a challenge dose of 1.5 Gy gammas. The results showed a significant decrease in the frequency of chromosomal aberrations [97]. Subsequent studies using comet assay showed a higher rate of spontaneous and induced DNA damage in the lymphocytes of the exposed group. Notably, the repair rate in the exposed group was higher as well, provided that the annual exposure was less than 10.2 mSv [98]. A more general study done by Tao *et al* looked at the mortality rate resulting from all cancers in a high background area in China, Yngjiang, which found a lower incident of cancer-related deaths in the mentioned area compared to normal background areas; this reduction however, was not significant [99].

Occupational adaptive responses of the lymphocytes of 12 hospital workers exposed to X and gamma rays investigated through isolation and a subsequent exposure to a 2 Gy irradiation showed lower incidence of dicentrics than the control group as shown in a study performed by Barquinero *et al* in 1995 [100]. A group of temporary nuclear power plant workers exposed to doses of 0-10 mSv showed no increase in the number of micronuclei. However, a Co-60 gamma dose of 3.5 Gy administered *in vitro* in high and low dose-rates (1 Gy/min and 4 mGy/min, respectively), significantly reduced the micronuclei frequency. Interestingly, the degree of adaptation was more significant after a low dose-rate challenge dose [101].

1.6.6.2- Adaptive Response Mechanisms

Ionizing radiation is not the only agent to induce adaptive responses. ROS, hydrogen peroxide, hyperthermia, and many other agents are known to cause adaptation in different living systems and organisms. Considering the fact that all these agents cause DNA damage, the phenomenon has been widely linked to this stimulus [50]. Adaptive responses have also been studied in the context of their connection with other phenomena like radiation-induced bystander effects and hypersensitivity [50,60,75,89,102–104]. Cellular signalling seems to play an important role in adaptation process which may include stress response and DNA damage repair [50]. There is also evidence for a role of cell cycle and distribution of cells within the cycle on adaptation results. This influence, however, is subject to great variability and therefore debate. A 20 mGy priming dose delayed the cell cycle progression in human ML-1 (Myeloblastic Leukemia) cells as shown by Amundson *et al* in 2001[105], while Aghamohammadi and Savage had previously shown that a lower priming dose of 10 mGy had no effect on cell cycle delays [106]. Research also suggests the involvement of DNA repair related proteins such as poly (ADP-ribose) polymerase (PARP), DNA dependent protein kinase (DNA-PK), ataxia telangiectasia mutated (ATM) and p53 [60,82,83,107–109].

1.7- Aims and Objectives

Radiation in low doses and smoking are both among stressors that humans are exposed to, both environmentally and medically. Many studies have looked into the

effect of nicotine on different cell lines and tissues to demonstrate the detrimental effects of nicotine. A plethora of published research can be found on radiation effects on cells, animals and other biota, many of which have focused on low dose and non-targeted effects. However, there is a considerable debate about the detrimental effects of exposure to ionizing and an ever growing concern about the severe health effects associated with smoking in developing and developed countries. Interestingly, not so many researchers have focused on the interaction between these two widely-affecting sources of public exposure. The work of Mothersill *et al* on nicotine and bystander effect triggered this study, but no other studies have so far investigated the combination of these effects.

This research strives to shed light on the impact of nicotine on the non-targeted effects of ionizing radiation. The focus is on understanding the interactions between nicotine exposure and two most important and well-studied non-targeted phenomena, namely, bystander effect and adaptive responses.

This thesis investigates the kinetics of nicotine both alone and in conjunction with low-dose radiation to better understand the behaviour of the drug on the model system used. Furthermore, it focuses on the impact nicotine on bystander effect through clonogenic survival assay, viability assay, and immunofluorescence. The impact of nicotine on adaptive responses is also investigated to explore whether nicotine can activate repair mechanisms to protect the cells against further damage caused by a separate stressor.

Understanding how these stressors interact with each other and relate to one another can provide an insight into health risks to the public and help towards a better modelling system for environmental and medical risk assessment and protection of the public.

Chapter 2

Materials and Methods

The materials, experimental procedures and protocols used in the course of the presented research are explained individually and in detail in this chapter. These include introducing the cell lines, chemicals and reagents, clonogenic assay technique, kinetics of nicotine in vitro, details of radiation induced bystander and adaptive response experiments, viability and immunofluorescence assay.

2.1- Cell lines

2.1.1- HPV-G cell line

HPV-G is a non-transformed skin keratinocyte cell line derived from neonatal human foreskin transfected with human papilloma virus [55]. These cells were originally received as a gift from J. Di Paolo, NIH, Bethedsa and maintained and cultured in our lab. Keratinocytes are the predominant cells in the epidermis layer of human skin. Papilloma viruses are small DNA viruses infecting epithelial cells in humans as well as other species, most of which cause benign lesions. Among the more than 70 Human Papilloma Viruses (HPV) identified, only a few are known to be malignant and associated with invasive squamous cell carcinoma and high grade interaepithelial

neoplasia [110]. HPV16 is in frequently found in cervical carcinomas and has been shown to increase the lifespan of keratinocytes to the extent that renders them immortal, which is more than 200 potential doublings (PD) [111]. This is achieved through co-expression of HPV16 E6 and E7 oncogenes that represses p53 gene without making the cells tumorigenic [112].

The appearance of the cell line grown in culture is in the form of monolayer with typical cobblestone pattern pertaining to epithelial cells as shown in Figure 2.1. The approximate doubling time of the cells is 22 hours [84].

Not all cell lines can produce radiation-induced bystander signal, nor do all cells respond to the signal produced by donor cells. HPV-G cells have been used in many studies as a reporter system because they both produce and respond to the bystander signal.

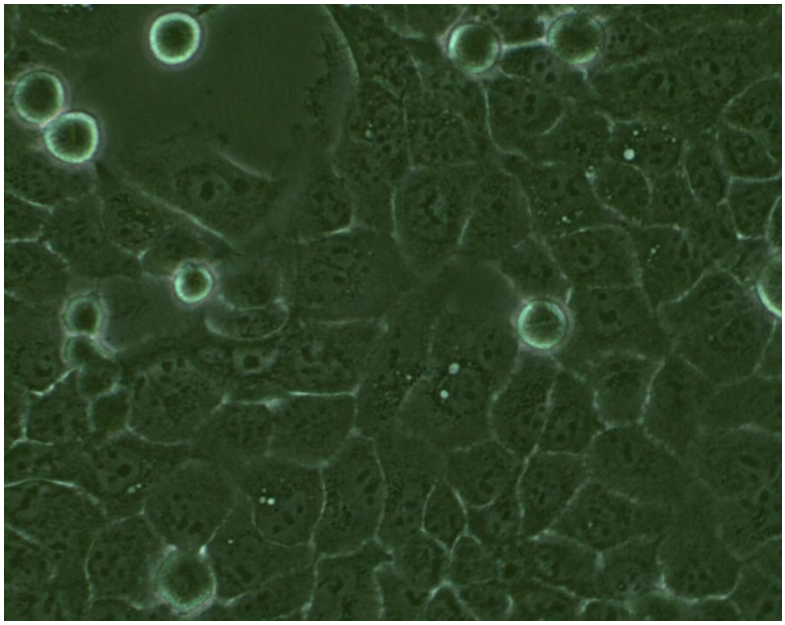


Figure 2.1- Phase contrast image of HPV-G cells in vitro (40X objective)

2.1.2- RT-112

RT-112 is a Homo sapiens (human) urinary bladder carcinoma cell line from Caucasian ethnicity. These cells belong to the category of epithelial cells which form a monolayer as shown in Figure 2.2. RT-112 is supplied by Cell Line Services, Germany and was a kind gift from Dr. Rub Bristow, Princess Margaret Hospital, Toronto, ON. Frozen cells were thawed and maintained in culture media explained in the following section. Cells were sub-cultured every 6-8 days as suggested by the cell line provider. RT-112 cells were tested for their bystander signal production and response and proved to be suitable for our experiments as shown in the following chapter.

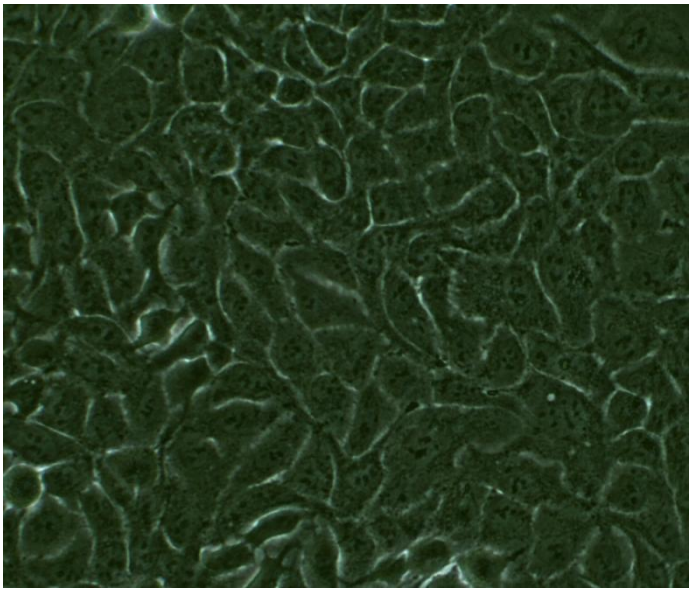


Figure 2.2- Phase contrast image of RT-112 cells in vitro (40X objective)

2.2- Cell Culture

All the reagents for cell culture were obtained from Gibco (Grand Island, NY) unless otherwise stated. All cell culture work and experiments were performed in a class II laminar flow cabinet. Cell stocks were maintained in T-75 flasks (250 ml) in 40 ml culture media. HPV-G cells were grown in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) (Invitrigen, Burlington, ON), 5 ml of Penicillian- Streptomycin (Gibco, Burlington, ON), 5 ml of L-Gluthamine (Gibco, Burlington, ON), 0.5 ug/ml hydrocortisone (Sigma-Aldrich, Oakville, ON), and 20 ml of 1M HEPES buffer solution (Gibco, Burlington, ON). All cells were incubated at 37°C and 5% carbon dioxide in air. RT-112 cells were kept in MEM alpha media supplemented with 10% FBS (Invitrigen,

Burlington, ON), 5 ml of Penicillian- Streptomycin (Gibco, Burlington, ON), 5 ml of L-Gluthamine (Gibco, Burlington, ON), and 20 ml of 1M HEPES buffer solution (Gibco, Burlington, ON) with the same incubation settings as HPV-G cells.

2.3- Irradiation

Cells were irradiated in T-25 flasks using McMaster University's Caesium-137 source. Caesium-137 decays to stable Barium-137 and emits 661.7 keV gamma rays as shown in Figure 2.3. All doses were delivered at the dose rate of 0.15 Gy/min, at the source to flask distance of 40 cm and a minimum field size of 40×40 cm.

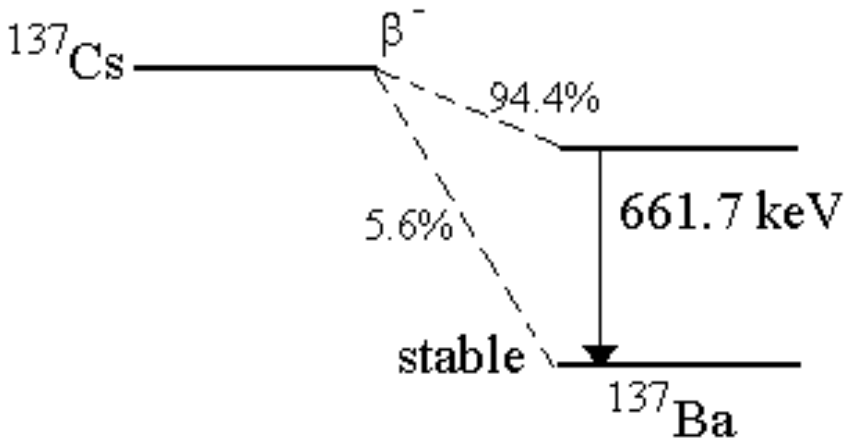


Figure 2.3- Decay scheme of Cs-137

2.4- Nicotine

Nicotine was purchased from Sigma Aldrich (Mississauga, ON) in the form of one gram of a water permissible liquid with 1.01 kg in 1 litre.

The original concentration was calculated to be 6 M which was diluted to 500 mM and was then serially diluted thereafter to achieve 50 mM to 50 nM stock dilutions in 10-fold steps. 0.1 ml of each of the stock concentrations was added to 5 ml of media in the flasks to give the final concentrations which were then used for various experiments.

2.5- Subculture

Cells growing on plastic continue dividing until the entire surface of the plastic is covered; at this stage they need to be removed and re-plated in a new flask or dish which is referred to as passaging or sub-culturing. The time between successive passages depends on the cell line and the number of cells seeded in the new flask.

For the purpose of experiments, T-75 flasks that were 90-100% confluent received a media change on the previous day in order to energize the cells. Cells were dislodged using Trypsin which serves to digest the extracellular matrix (ECM) produced by the growing cells, supplemented by EDTA to help break the calcium dependent intercellular junctions [113]. Cells were rinsed with the aforementioned solution once to remove the traces of medium which interfere with the action of Trypsin, followed by

incubation with 10 ml of Trypsin/EDTA for no more than 10 minutes to ensure the smooth detachment of the cells from the plastic bottom of the flask. The Trypsin/cell suspension was then neutralized with 10 ml of the culture medium and pipetted gently and repeatedly to obtain a single-cell suspension which in turn would ensure an accurate cell count and seeding. 1 ml of the suspension was added to a new flask filled with 40 ml of fresh culture media while the original flask with the cell suspension residue was kept as backup.

To be able to seed the exact number of cells required for each experiment, 1ml of the stock cell suspension was diluted in 10 ml of Isoton II buffer and counted three times using a coulter counter machine preset to gate the size of HPV-G cells, the mean of which, after background subtraction, gave the number of cells in 1 ml of the cell stock. The stock was thereafter serially diluted to allow for more accurate number of cells to be plated.

For this purpose, a coulter counter machine was used. The setting of the counter is such that gauges the size of the cells to be counted. In each case, 1 ml of the stock cell suspension was diluted in the Isoton II buffer required for counting. The unit operates on the basis of the changes in electrical impedance. The particles suspended in the buffer solution act as small insulators as they pass through the aperture changing the impedance of the aperture. The result is an electrical charge which the frequency and amplitude of which depends on the number of particles in the sample and the volume of the particles, respectively. To assure a reliable cell count, the aperture size is chosen based on the

average diameter of the cells to be counted to allow for the passage of only one particle at a time. The occasional coincidence, which is presence of multiple particles within the aperture, is corrected by the instrument through its statistical dependence on sample concentration [114].

2.6- Nicotine kinetics

In order to investigate the kinetics of nicotine in vitro, flasks were set up and divided to two groups of irradiated and controls with varying concentrations of nicotine. In each group nicotine was removed after 2, 6, and 24 hours, or left in the culture media for the entire period of incubation. The irradiated group was irradiated to a low dose of 0.5 Gy gammas one hour post exposure to nicotine. Both groups were then incubated for 10-14 days at which point they were stained and colonies were scored.

2.7- Radiation-induced Bystander Experiment

Donor flasks were set up at the density of 10^5 cells per flask to produce a strong bystander signal. The reporter or recipient flasks were plated at the density of 500 cells per flask. 6 hours post plating, the incubated donor flasks were exposed to gammas of 0.5 Gy and returned immediately to the incubator for 1 hour, after which the irradiated cell conditioned medium (ICCM) or control cell conditioned medium (CCCM) was harvested from the donor cells and transferred to the recipient flasks, the medium from which was discarded right before the transfer. The recipient flasks were then incubated for 10-14

days, followed by staining and scoring of the colonies. In order to be certain that the effect observed is solely due to the ICCM and CCCM and not the donor cells, the harvested media from the donor flask was filtered using a 0.22 μm filter. This sterilizes the media and insures that no donor cells are present in the transferred media.

A 1 hour incubation time was chosen based on the study that showed incubation times between 30 minutes to 24 hours did not alter or diminish the bystander signal [73].

2.8- Nicotine and Radiation-induced Bystander Effect

The details of this experiment are similar to the bystander protocol explained above with the exception that one hour prior to irradiation varying concentrations of nicotine were added to the donor flasks.

In a separate experiment, after following the steps explained in this and also the previous section, the ICCM and CCCM were removed one hour after transfer to the recipient flasks and replaced with fresh media to study the effect of short term exposure of the bystander flasks to nicotine. Both experiments were incubated for 10-14 days; colonies were stained and counted, subsequently.

2.9- Effect of the Sequence of Exposure to Nicotine and Radiation

To be able to study and compare the effect of presence of nicotine at the time of exposure to radiation, two experimental groups were set up with identical number of cells and incubated for 6 hours. After the incubation period, the first group was exposed to nicotine concentrations and then exposed to radiation doses of 0.1- 5 Gy after 18 hours of incubation. The second group was irradiated to the same doses of radiation post initial incubation and nicotine was added to the flasks immediately after irradiation. Both groups were incubated for 10-14 days and the colonies were stained and scored.

2.10- Adaptive Response

In this experiment cells were plated with the density of 1000 cells per flask and incubated for 6 hours to allow the cells to adhere to the bottom of the flasks. The number of cells plated was decided based on the radiation dose delivered. After 6 hours, varying concentrations of nicotine were added to the flasks as the priming stressor. Controls for low a dose of radiation (0.1 Gy) as the primary stress and also the effect of challenge dose alone were included. After exposure to the priming stressor, be it radiation or nicotine concentration, the cells were returned to the incubator and incubated at 37 degrees for 3 and 12 hours prior to exposure to the challenge dose of 5 Gy. Thereafter, the flasks were incubated for 10-14 days to allow the colonies to form, stained and counted subsequently.

2.11- Viability Assay

Alamar Blue (Gibco, Burlington, ON) was used to stain the cells for viability. Live cells maintain a reducing environment within the cytosol. Alamar Blue takes this fact to advantage. The active ingredient of the dye, Resazurin, is non-toxic, cell permeable and blue in nature with no fluorescence. When this compound is taken up by the viable cells, it is reduced to resorufin. Resorufin is fluorescent and red in color. Viable cells continuously convert resazurin to resorufin which can be measured through fluorescence or absorption [115].

Recipient and donor cells were plated in 24 and 96-well plates with suitable densities, followed by exposure to nicotine and subsequently radiation. The bystander protocol was followed as explained in previous sections. One hour post media transfer, Alamar Blue was added to the wells without further dilution. The reagent was added at 10% of the sample volume (10 µl Alamar Blue for 100 µl of sample), wrapped in aluminum foil to protect the cells from light and incubated for 4 hours in 37° C. The absorbance was measured using a plate reader (Molecular Devices, model: spectra MAX 340PC) at 570 nm. A reading was also made as 600 nm to be used as reference. Using only media in the wells, a background absorbance measurement was also made and deducted from the sample measurements.

2.12- Immunofluorescence Assay

Donor cells were plated in standard 24-well plates (Falcon, Franklin lanes, NJ) and recipients in the glass bottom 96-well plates (Greiner bio-one, Germany) to provide better images. Recipient cells were incubated for 24 hours as opposed to the usual 6 hours to avoid losing cells during immunofluorescence preparation due to multiple rinsing. Following the nicotine bystander experiment, the recipient cells were fixed for 3 minutes with 4% fresh depolymerised paraformadehyde. To visualize the membrane-associated nAChR subunits 7% sucrose was added to the fixing solution to avoid cell permeabilization. Cells were then rinsed twice and incubated over night at 4°C with rat monoclonal antibody against nicotinic acetylcholine receptor subunits α1, α3, and α5 (Abcam, USA) as the primary anti-nAChR subunit antibody. Binding of the primary antibody was visualized by incubating the cells with FITC-conjugated goat anti-rabbit IgG antibody (Invitrogen, Oakville, ON) for one hour in room temperature. The specificity of the antibody was confirmed by omitting the primary antibody. After rinsing, the cells were examined using a fluorescence microscope (Olympus 1X81) using ImagePro software and images were taken. The images were then imported and intensity values of the regions of interest were extracted using ImageJ software. The intensity of fluorescence was calculated pixel by pixel by dividing the summation of fluorescence intensity of all pixels by the area occupied by the pixel for each region of interest. Samples of cell-free areas were also measured for the purpose of background subtraction.

3 different cells were chosen from each of the triplicate wells and also 3 background areas were picked for each sample.

CHAPTER 3

RESULTS

3.1- Kinetics of Nicotine in HPV-G Cells

Nicotine kinetics can vary to a remarkable extent depending on the cell line studied. In order to investigate the time-dependent effects of nicotine on HPV-G cells and also any modifying effects if used with radiation, a total of 78 T-25 flasks were set up with a density of 500 cells per flask. Flasks were divided into two groups and incubated for 6 hours to allow the cells to adhere to the bottom of the flasks. After the first incubation period, nicotine was added to the flasks in both groups in concentrations of 10 nM, 100 nM, and 1 μM (consistent with the reported blood and plasma nicotine levels [9]). At this point, group one was left in the incubator and triplicates from each concentration were taken out after 2, 6, and 24 hours and received a media change as to remove the nicotine and returned to the incubator subsequently. The last set of concentrations in this group received no media change and maintained the nicotine for the entire period of incubation. The second group was irradiated to a low dose of 0.5 Gy one hour post exposure to nicotine. Upon return, this group was also incubated and received the same media change regimen as the first group. Both groups were then incubated for 10-14 days at which point they were stained and colonies were scored.

Figures 3.1 and 3.2 show the results of the experiment for zero and 0.5 Gy doses for different concentrations. As can be seen in the figures, in both cases (i.e. irradiated and control) the pattern was consistent for 10 nM and 1000 nM as survival dropped in the first 6 hours and picked up again until it peaked at 24 hours and then decreased as the duration of exposure increased. For 100 nM concentration, however, both 0.5 Gy and zero doses, exhibited a different pattern, where survival increased up to 6 hours and then gradually decreased. Another interesting point is that the overall survival except for long exposures was higher than the control indicating that proliferation was enhanced.

Figures 3.3, 3.4, and 3.5 represent the same data in terms of control versus irradiation for each of nicotine concentrations. As depicted in the figures, the patterns for control and 0.5 Gy dose are completely consistent. Survival significantly decreased when cells were exposed to radiation for 10 and 1000 nM (table 3.1). Surprisingly, at 100 nM, exposure to radiation had no significant effect on survival of the colonies.

3.2- Prolonged Exposure of HPV-G Cells to Nicotine

Even though nicotine is cleared by the liver with a half life of approximately 120 minutes [9,10], a regular smoker is still chronically exposed to low concentrations of nicotine due to the boost provided by each smoking session. To investigate the effect of chronic exposure to nicotine, HPV-G cells were exposed to a wide range of nicotine concentrations 6 hours post plating and incubated for 10-14 days. The results of the stained and scored colonies are illustrated in Figure 3.6. An increase in the number of

colonies above the control level was observed for 100 nM and 10 μM in a bimodal fashion. This shows that at least in certain concentrations, nicotine protected the cells against death.

3.3- Effect of the Sequence of Exposure to Nicotine and Radiation

To investigate the possible correlation between the sequence of exposure to nicotine and radiation and the observed effect, a limited range of concentrations was chosen. Two experimental groups were set up with identical number of cells (500 cells per flask) and incubated for 6 hours. After the incubation period, the first group was exposed to nicotine in concentrations of 1 nM, 10 nM, and 100 nM and then exposed to radiation doses of 0, 0.1, 0.5, 1, 2, 3, and 5 Gy after 18 hours of incubation. The second group was irradiated to the same doses of radiation post initial incubation and nicotine was added to the flasks immediately after irradiation. Both groups were incubated for 10-14 days and the colonies were stained and scored. The results of this experiment are shown in Figure 3.7. For all concentrations of nicotine investigated in this experiment, the presence of nicotine at the time of irradiation enhanced the growth and survival of the colonies. For each of the concentrations, the pattern of response of the two groups was quite similar.

3.4- Nicotine and Radiation-induced Bystander Effect

The main body of the present research focuses on the impact of nicotine on radiation-induced bystander effects. A total of 78 T-25 flasks were plated for each of the radiation doses (0.1, 0.5, 1, 2, 3, and 5 Gy), half of which were plated as donor flasks with the density of 500 cells per flasks with the density of 100,000 cells and the rest as reporters or recipients. Cells were exposed to nicotine one hour pre irradiation and the ICCM or CCCM was transferred from donors to reporter cells one hour post irradiation. The results are shown in Figures 3.8 to 3.11. The observed effect was of a bimodal nature as shown in Figure 3.8, increasing and decreasing depending on the dose of radiation and concentrations used. Two distinguishable patterns were observed following high and low doses of radiation in Figure 3.8 and these are shown separately in Figures 3.9 and 3.10. Figure 3.9 depicts the pattern for low doses for which the survival was clearly decreased for 0.5 Gy when compared to 0.1 Gy. This effect was more prominent at higher concentrations of nicotine. The differences were especially significant for concentrations of 100 nM, 1 μM, 10 μM, 100 μM ($P<0.04$). Also shown in this figure is the bimodal effect of the combination of nicotine and RIBE. Doses of 1 Gy and higher exhibited the same bimodal response (Figure 3.10); however, the shape of the response was different than that of the low doses, with the rise and falls being more pronounced. When data was arranged in the form of dose-response curves for all concentrations, two separate patterns for the two lowest concentrations (0.1 nM and 1 nM) and higher concentrations (> 10 nM) was observed which is shown in Figure 3.11.

In a separate experiment, the effect of duration of exposure to nicotine in on RIBE was tested to see whether removing nicotine from the culture media of the donor cells prior to irradiation influenced the response of the cells when compared to prolonged exposure to nicotine previously investigated. To accomplish this, nicotine concentrations of 1, 10, and 100 nM were added to the donor flasks 5 hours post plating and subsequently removed after an hour, at which point the donors were irradiated. Figure 3.12 compares the results of short and prolonged exposure to nicotine in the context of radiation-induced bystander effect. It was shown that a short exposure to nicotine inhibited the survival and subsequent growth of the colonies. This inhibition was significant for all concentrations tested ($P<0.02$).

Small differences in the detailed analysis of serum batches seem to have a significant impact on the behaviour of cells and their response to bystander signal in vitro. Three different batches of FBS were tested to study the presence of such differences, the results of which is depicted in Figure 3.13. Clearly, significant differences were observed with respect to clonogenic survival of the cells. Furthermore, the consistency of the patterns of the responses was confirmed as illustrated in Figure 3.14.

3.5- Viability Assay

Alamar Blue is a simple and convenient method for assessing the viability of cells after a certain treatment. Donor cells plated (16,000 cells per well) in triplicates in 24-

well plates were exposed to nicotine after reaching confluency and were incubated for 24 hours, washed with PBS and received new media, after which they were irradiated to doses of 0.5 and 5 Gy as well as control for dose. One hour post-irradiation the ICCM or CCCM was transferred to the recipient cells plated with the density of 10,000 cells per well. Alamar blue was added to the wells at a volume of 10 μL for 100 μL of culture media without any dilution. No-cell controls were also included which served as background reading. Cells were then incubated in a cell culture incubator for 4 hours to reach maximum absorbance after which the absorbance was measured using a plate reader at 570 nm. Reading was also done at 600 nm as a reference for normalization. The results of this experiment are shown in Figure 3.15. The viability of cells decreased significantly with increasing dose of radiation when cells were exposed to nano-molar ranges of nicotine and increased with increasing dose of radiation in micro-molar ranges with one exception being 100 μM, for which the viability of cells increased when exposed to 5 Gy gammas comparing to 0.5 Gy. Interestingly, simple exposure to radiation exhibited an increasing trend as the dose of radiation increased.

3.6- Immunofluorescence Assay

Immunofluorescence is probably the most reliable way for observing and quantifying the expression of different receptors. Quantifying the expression of receptors is of vital importance when studying the impact of nicotine on a certain model system. To achieve this goal, the recipient cells were set up in special glass-bottom 96-well plates to achieve better image quality with density of and 500. Donor cells were plated in standard

24-well plates with the density of 1000 cells per well. These numbers were determined based on trial and error to achieve the necessary number of cells for visualization under the microscope. The recipient cells were incubated for 24 hours before treatment. This was done to ensure that cells had adhered to the bottom of the wells as the previous trial had shown that a majority of cells were removed during numerous washings required by the protocol. 100 μL of 4% paraformadehyde containing 7% sucrose was added to the recipient cell for 3 minutes to fix the cells. Sucrose was added in order to avoid cell permeabilization as nAChRs are extracellular receptors with the epitope being on the extracellular side. The fixed samples were washed and incubated over night at 4°C with 50 μL of rat monoclonal nicotinic acetylcholine receptor antibody against alpha 1, 3, and 5 subunits (Abcam, USA). The choice of antibody was based on the published studies [5,20]. To visualize the binding of the receptor FITC-conjugated goat anti-rabbit IgG antibody was used as the secondary antibody. 50 μL of the secondary antibody was added to the cells, one hour after which the samples were examined under the microscope. Nicotine caused an over expression of the receptor at concentrations of 100 nM and 1000 nM ($P=0.038$ and $P=0.015$, respectively) with no significant increase at 10 nM compared to the control ($P=0.805$). The number of receptors increased significantly for 10 nM ($P=0.033$) and 100 nM ($P=0.032$) when cells received the ICCM from the donor cells. The effect, however, was opposite for 1000 nM where a statistically significant decrease was observed. This decrease however was only significant when compared to nicotine data and not the control ($P=0.0003$ and $P=0.922$, respectively). When compared to nicotine-only intensities, the bystander group showed a significant increase at 10 nM

(P=0.042) followed by a drop at 1000 nM (P= 0.0003) and 100 nM which failed to reach significance (P= 0.558) (figure 3.16).

Figure 3.17 is an illustration of immunofluorescence images of HPV-G cells taken with the Olympus 1X81 microscope at 20X magnification. The images were tinted and enhanced for the purpose of illustration.

3.7- Nicotine and the Adaptive Response of HPV-G cells

To investigate whether or not nicotine can act as a priming stressor to induce an adaptive response, T-25 flasks were set up at a density of 1000 cells per flask and received either 100 mGy or varying concentrations of nicotine (1-1000 nM) as a priming stressor; this was followed by 3 and 12 hours of incubation after which all the flasks were exposed to a challenge dose of 5 Gy to investigate whether the length of time gap between the priming and challenge stressor had an effect of the response of the cells. The results are shown in Figure 3.18 for 3 hours and 12 hours of time gap, respectively. It was observed that increasing the time between priming and challenge stress had no effect on the survival of the cells. A slight decrease in survival was observed in cells exposed primarily to 100 mGy or nicotine when compared to the survival of the cells which received 5 Gy only and no priming stress. This decrease was not significant for 12 hours (P>0.22). In the 3 hours group however, this decrease was significant for 100 nM and 1000 nM (P=0.035 and P=0.027). The survival of the cells that received nicotine was lower than those exposed to 100 mGy, but this decrease failed to reach statistical

significance in the 12 hour experiment (P>0.39), however, it was statistically significant for 100 nM and 1000 nM in the 3 hour group (P=0.022 for both).

3.8- RT-112 Cells and Radiation-induced Bystander Effect

The same protocol used with HPV-G cells was employed to investigate whether or not RT-112 cells showed bystander signalling following irradiation. The results as depicted in Figure 3.19 indicated that RT-112 cells generate and respond to bystander signal as the bystander survival was significantly lower than the controls and also 0.5 Gy irradiated flasks (P=0.0002). The controls for sham irradiation and also the irradiated media remained at the control levels, confirming that results observed were solely due to the factors released from the cells into the culture media and not the media or other external factors.

3.9- Nicotine and Radiation-induced Bystander Effect in RT-112 Cells

In an experiment similar in details to HPV-G cells previously explained in section 3-4, the impact of nicotine on RIBE on RT-112 cells was studied. The concentrations of nicotine, however, were limited to 10-1000 nM, controls for sham irradiation (absolute control), media irradiation were also included as usual. Results of this experiment showed that survival of the cells increased with the increasing concentration of nicotine from 10

nM to 1000 nM (Figure 3.20). Zero concentration of nicotine remained at the same level as the control. Sham irradiation and also media were also in agreement with the controls. Surprisingly, exposure to 0.5 Gy gammas had no significant effect on the average survival of the cells, however, the errors were relatively high (±13%).

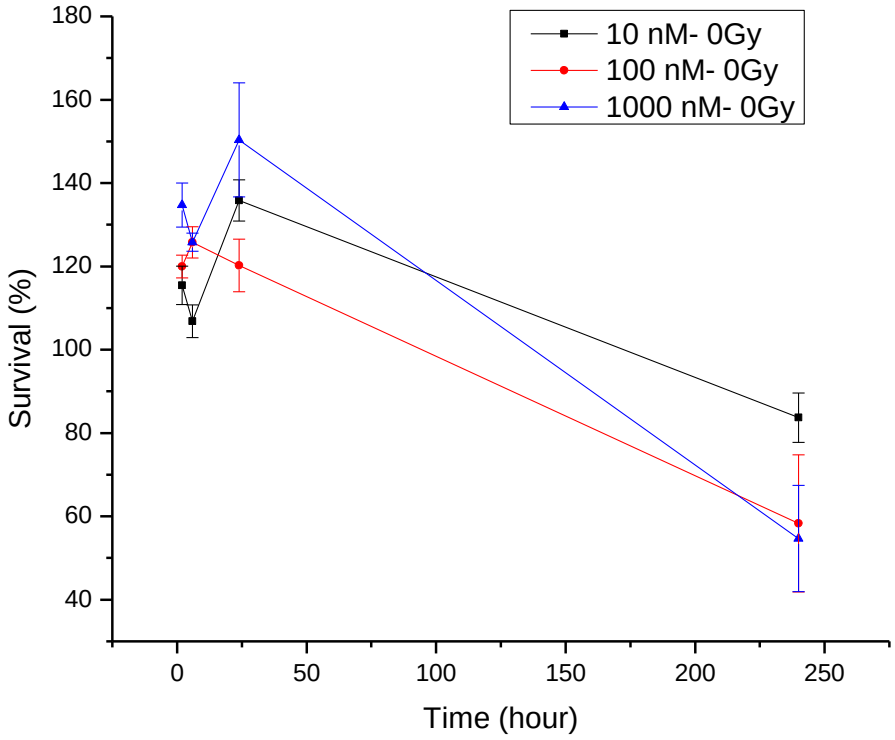


Figure 3.1- Kinetics of nicotine for the control group i.e. no irradiation. It can be seen that the pattern for 10 nM and 1000 nM are consistent, whereas at 100 nM there exists a remarkable variation in the first 6 hours

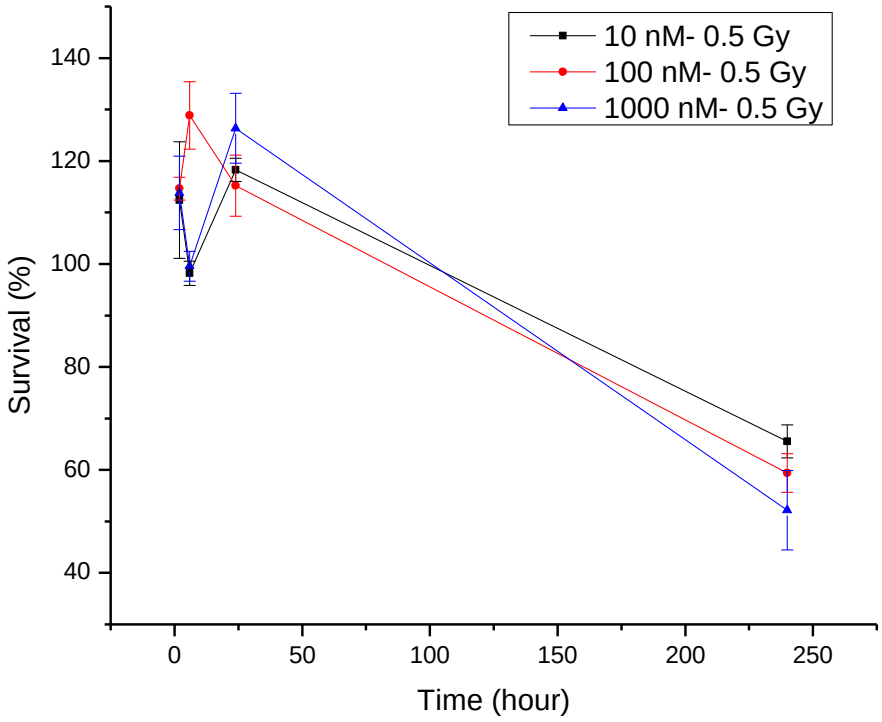


Figure 3.2- Kinetics of nicotine for the irradiated group. Flasks were irradiated at 0.5 Gy. A deviation from the pattern is clearly observed in the 6 hour time frame for 100 nM of nicotine

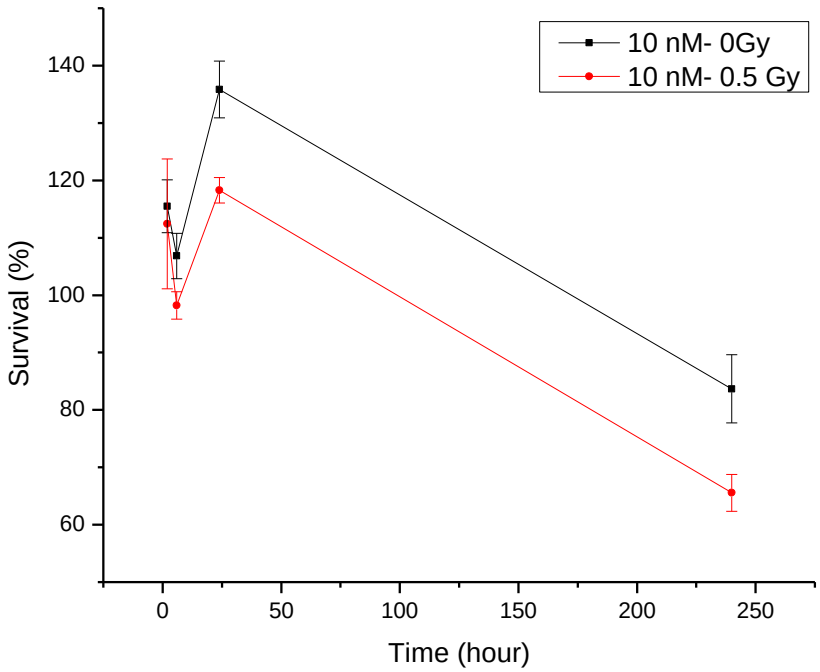


Figure 3.3- Kinetics of 10 nM of nicotine for control and irradiated conditions. The irradiated group closely followed the control group with a significant decrease in survival

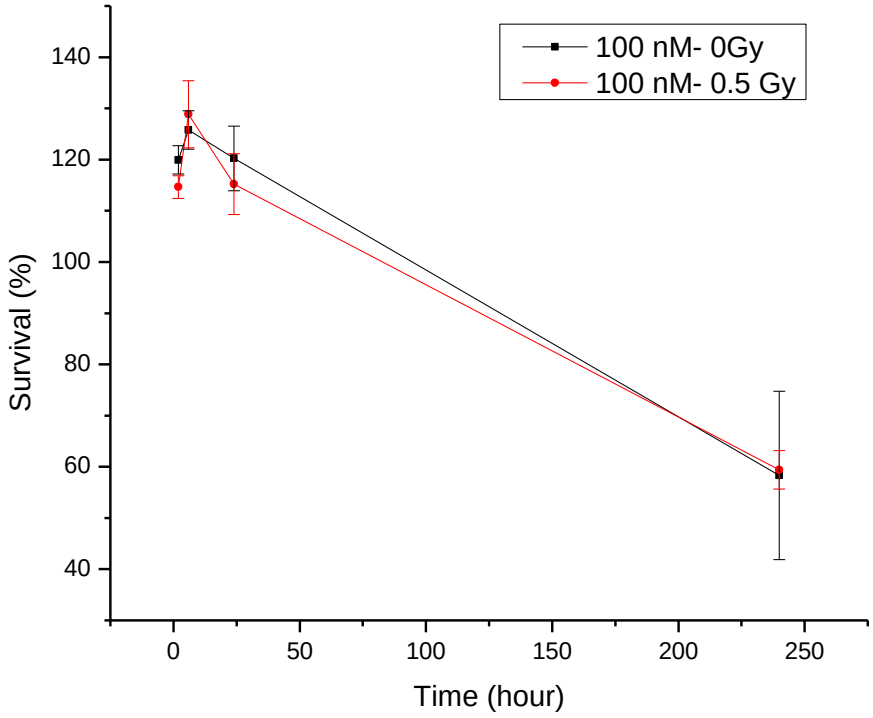


Figure 3.4- Kinetics of 100 nM of nicotine for control and irradiated conditions. No significant difference was observed in the irradiated group with respect to the controls

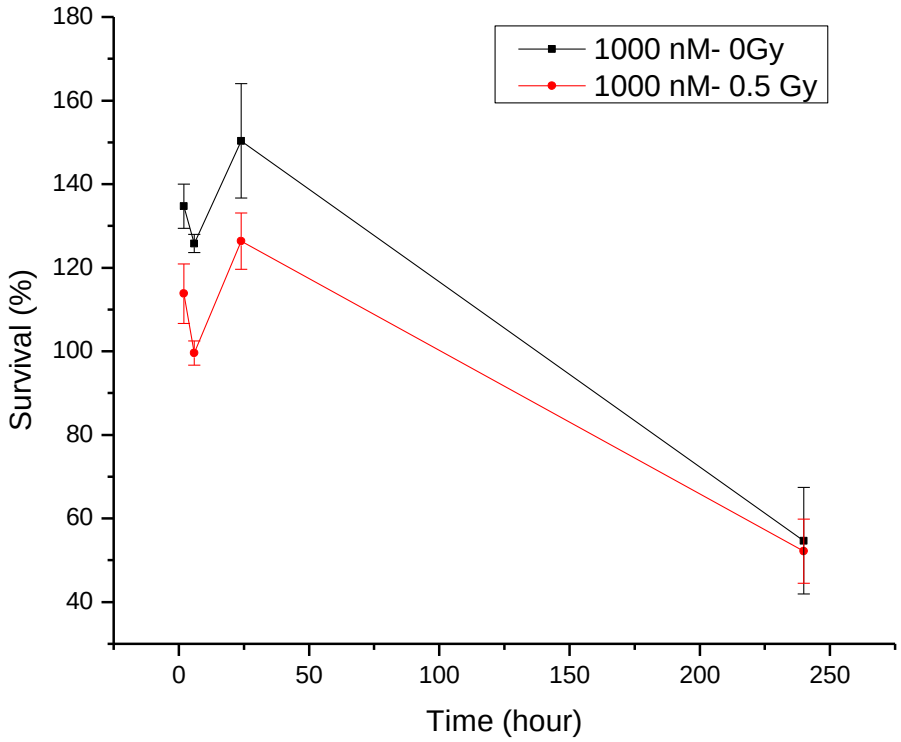


Figure 3.5- Kinetics of 1000 nM of nicotine for control and irradiated conditions. A significant decrease in survival for the irradiated flasks which follows the pattern of the control group was observed

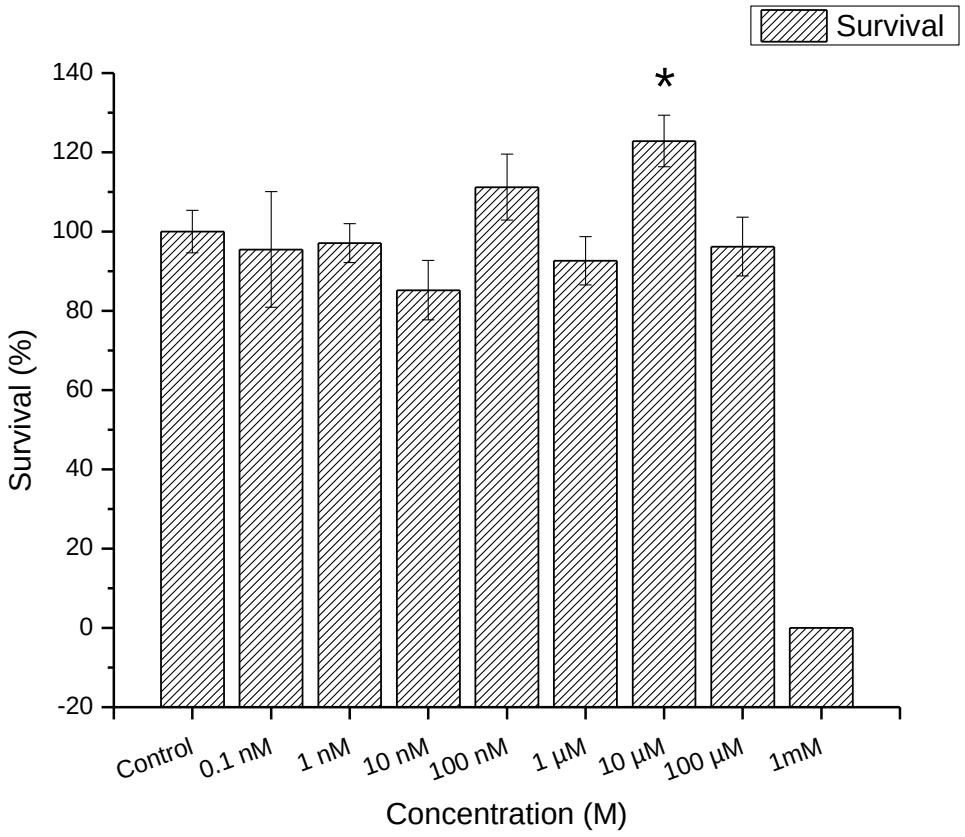


Figure 3.6- Survival after chronic exposure to various nicotine concentrations. Survival is elevated with respect to control group for concentrations of 100 nM and 10 µM, the increased levels, however, are not significant for 100 nM

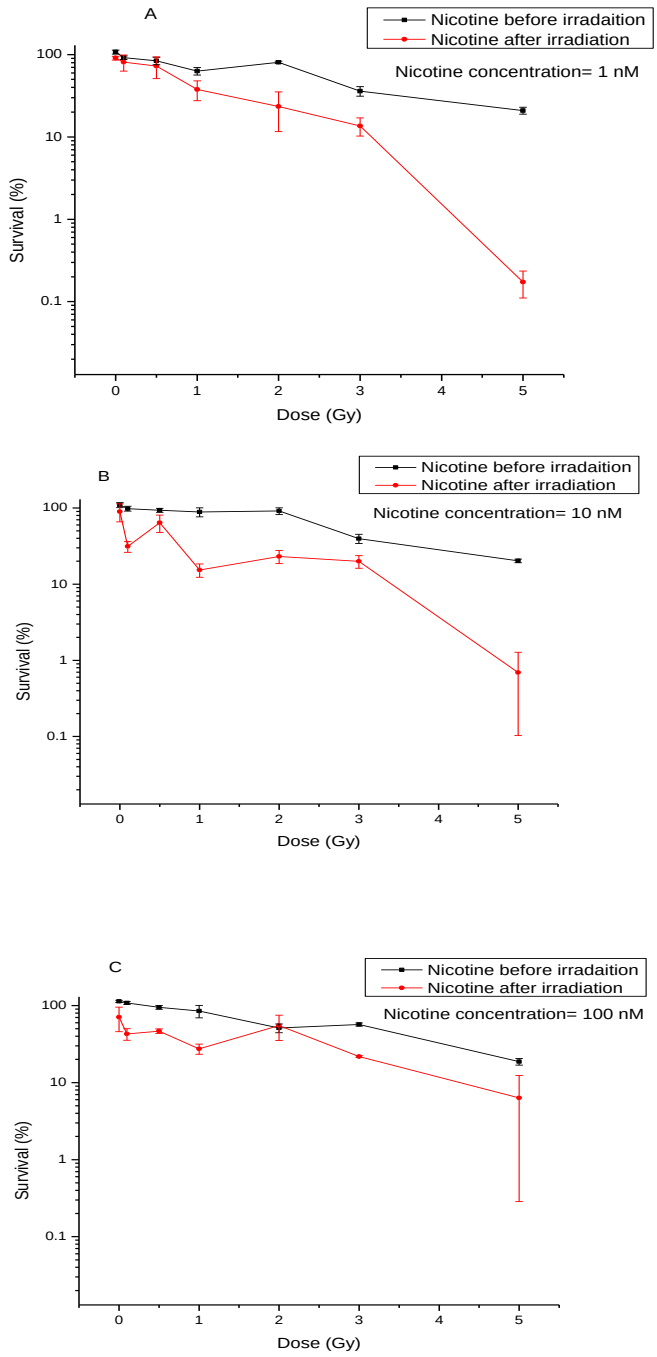


Figure 3.7- The effect of sequence of exposure to nicotine and radiation. For all concentrations the presence of nicotine at the time of irradiation enhanced the survival

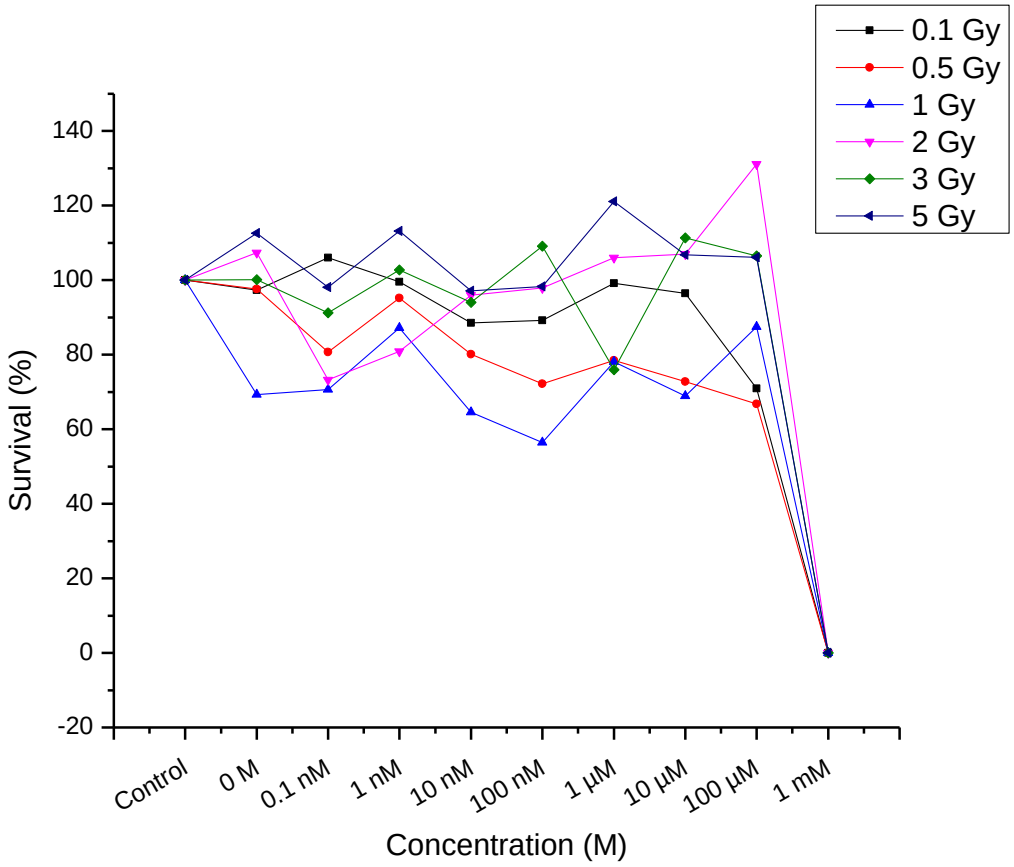


Figure 3.8- Impact of nicotine of radiation-induced bystander for various gamma doses. Two distinct patterns were identified. The error bars were omitted to avoid complicating the figure

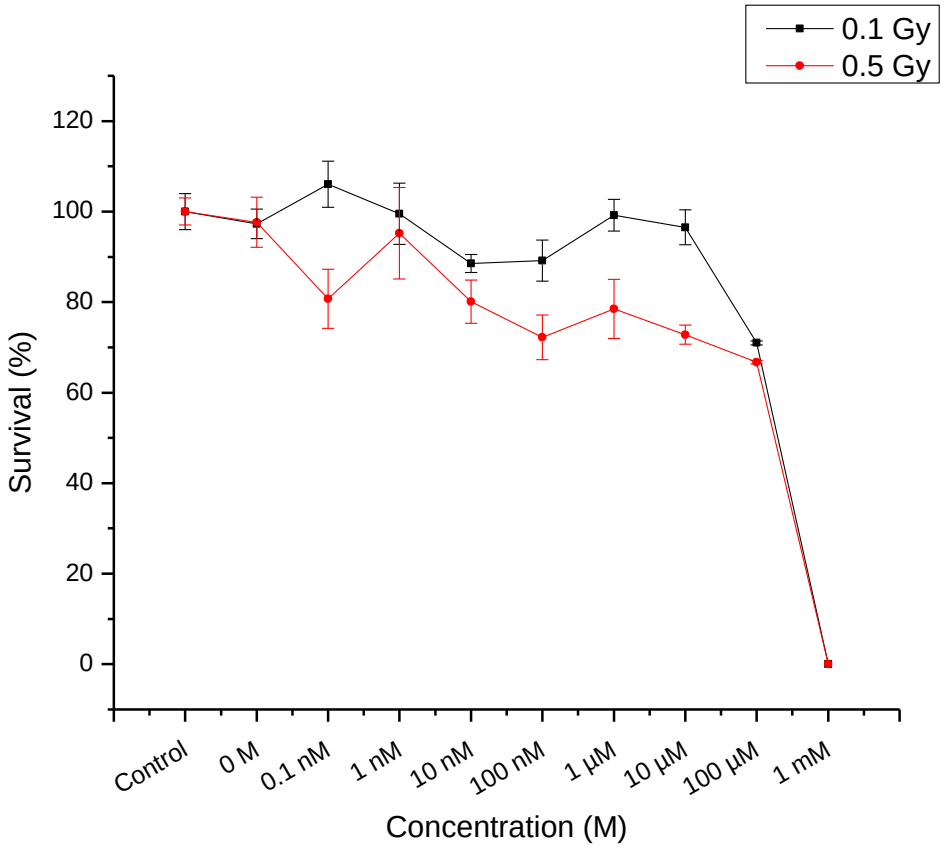


Figure 3.9- The consistent pattern of response for low doses of radiation indicating a bimodal response

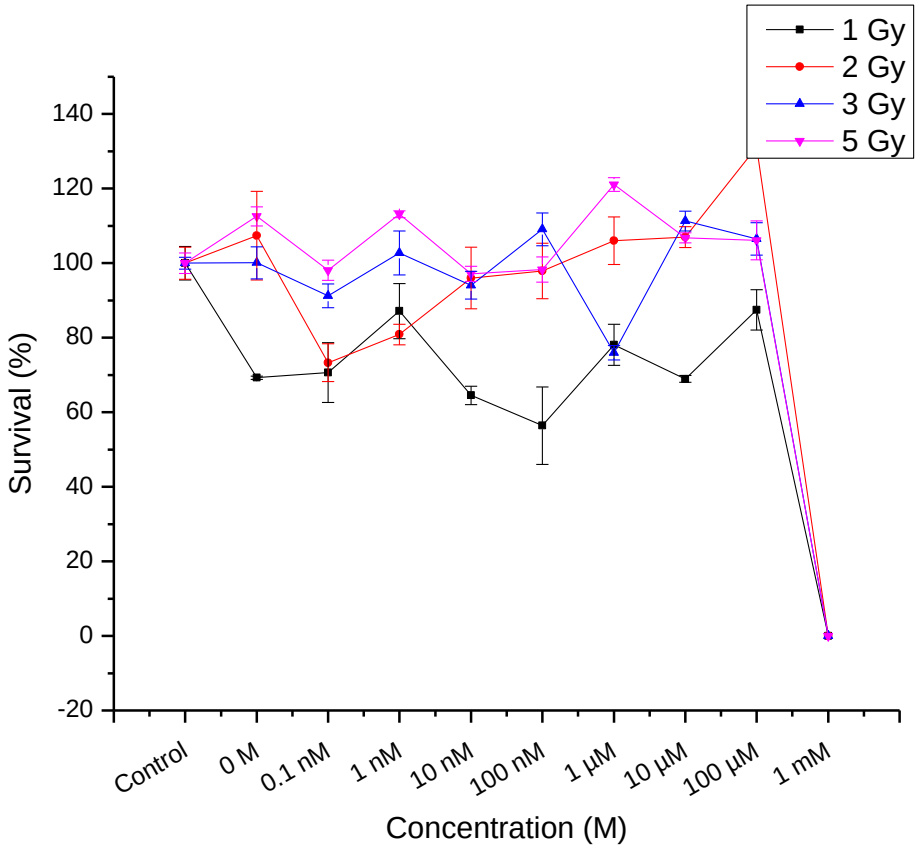


Figure 3.10- The consistent pattern of response for high doses of radiation indicating a bimodal response

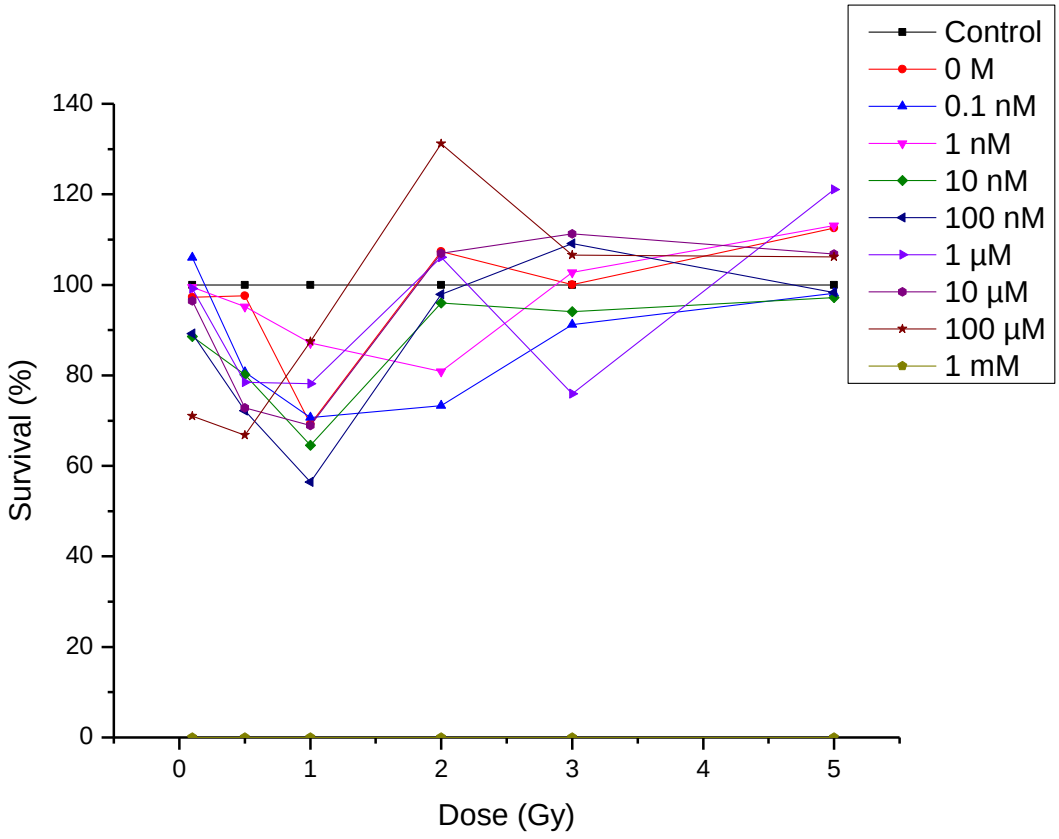


Figure 3.11- Schematic of similarities in the patterns of response for 0.1 nM and 1 nM. The rest of the concentrations also follow a common pattern, however less uniform than those of the low concentrations

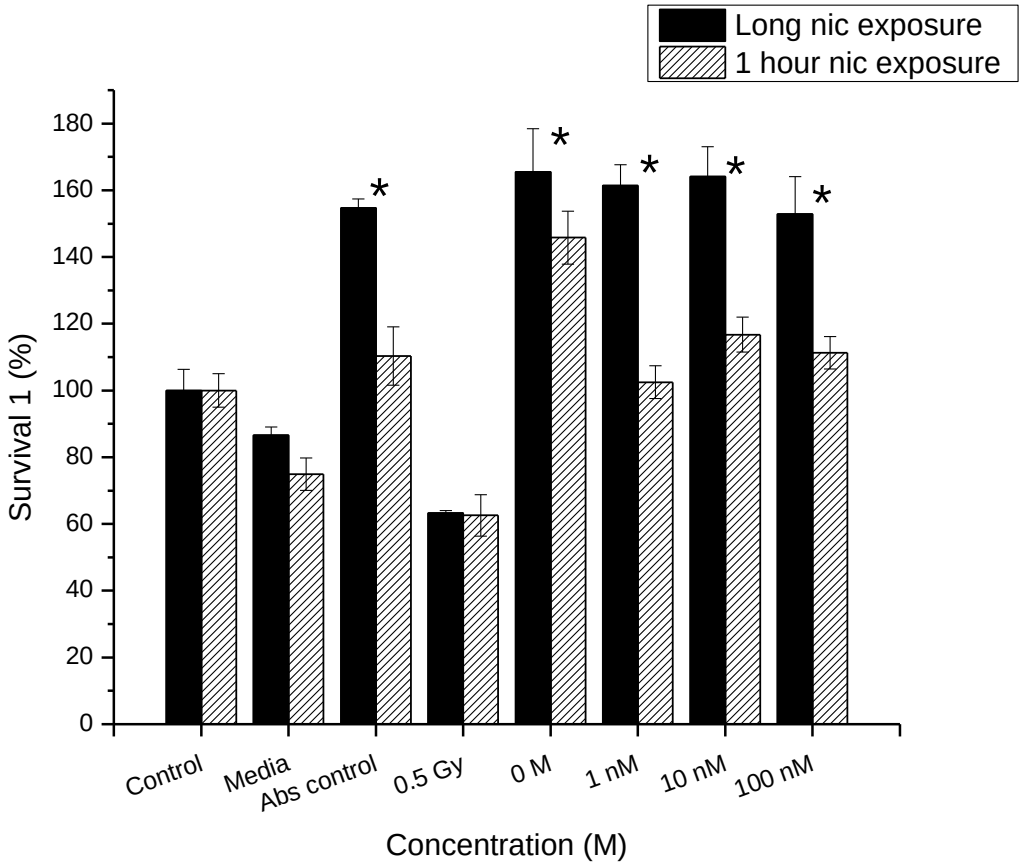


Figure 3.12- Comparison of the effect of short vs. prolonged exposure to nicotine on radiation-induced bystander signal. Acute exposure decreased the survival of the colonies to a significant extent

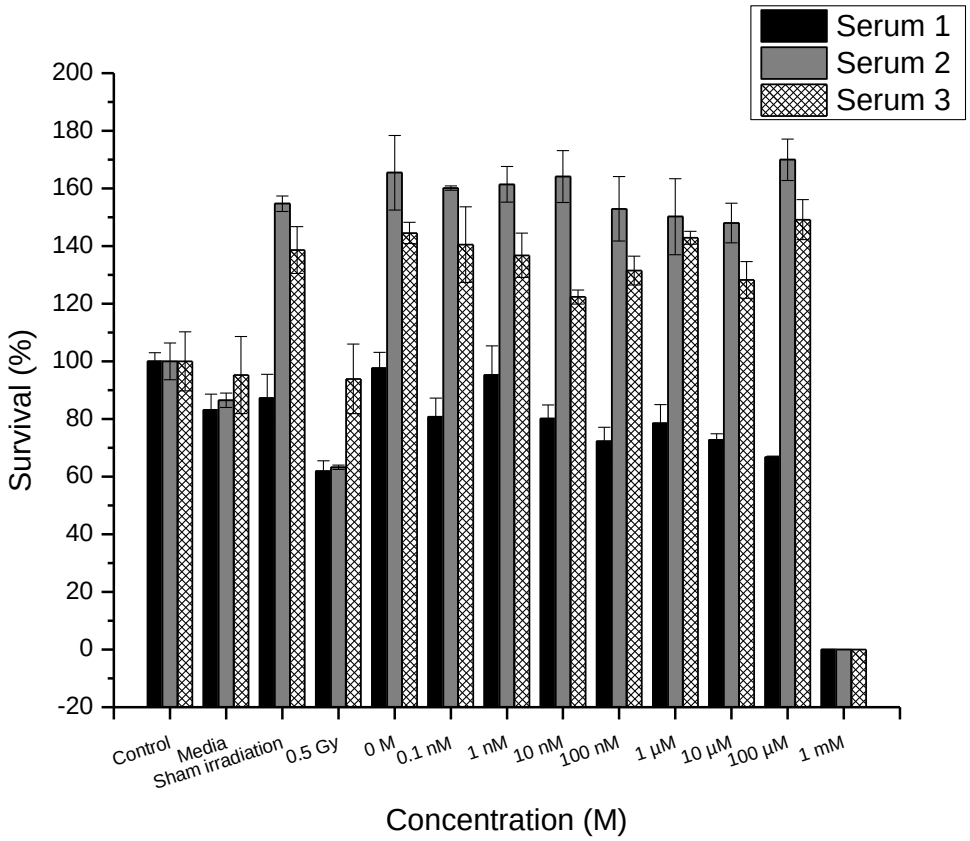
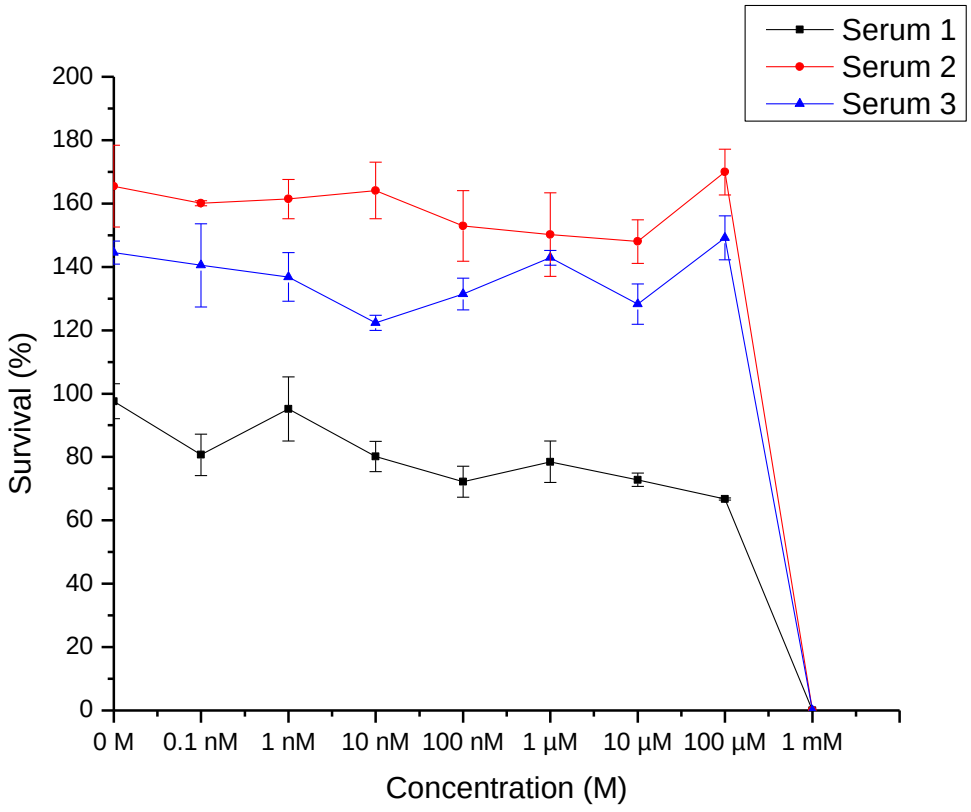


Figure 3.13- Comparing the effect of various batches of FBS on the survival of the bystander cells exposed to a wide range of nicotine concentrations. Significant variations were observed for each of nicotine concentrations with an overall consistent response throughout the nicotine range



3.14- An alternative representation of Figure 3.12 to further illustrate the consistent pattern of response for different batches of FBS

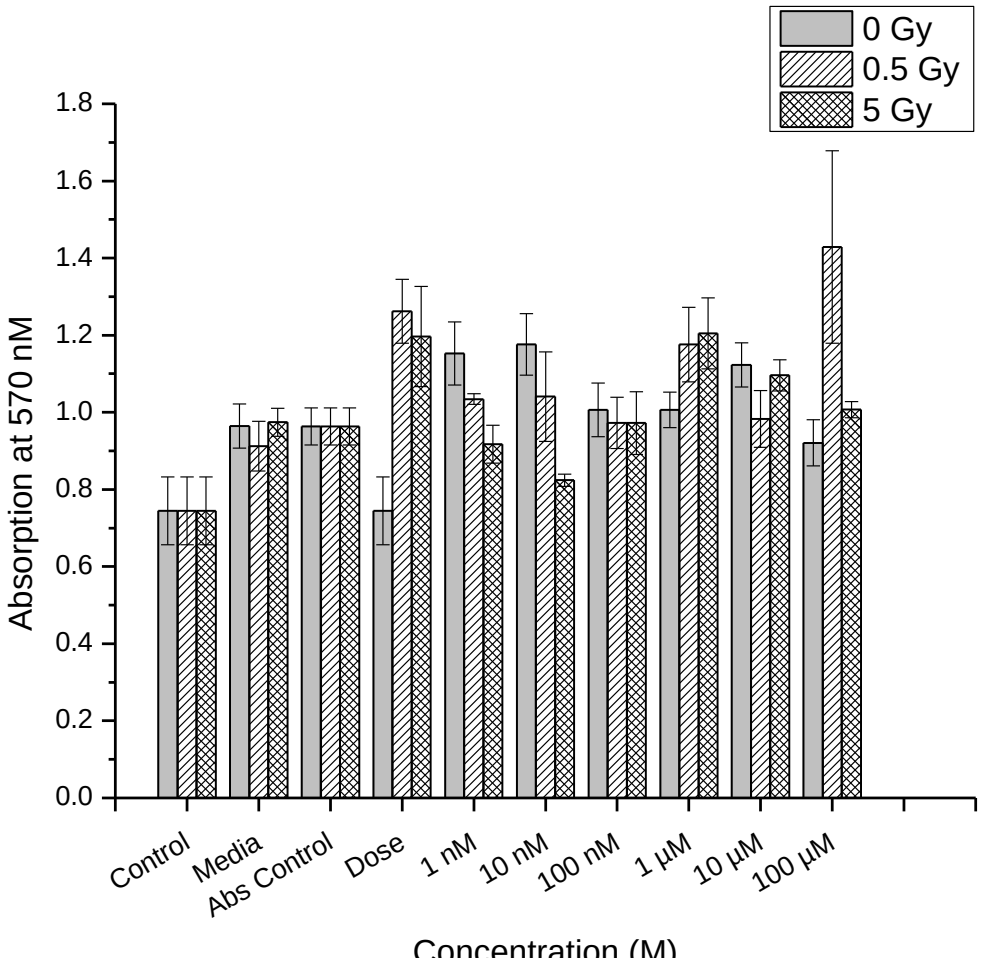


Figure 3.15- Schematic of viability assay results. Viability decreased with increasing dose of radiation for nano-molar ranges. In the micro-molar ranges no significant correlation between viability and radiation dose was observed. Interestingly, viability increased when cells were exposed to radiation alone compared to the controls

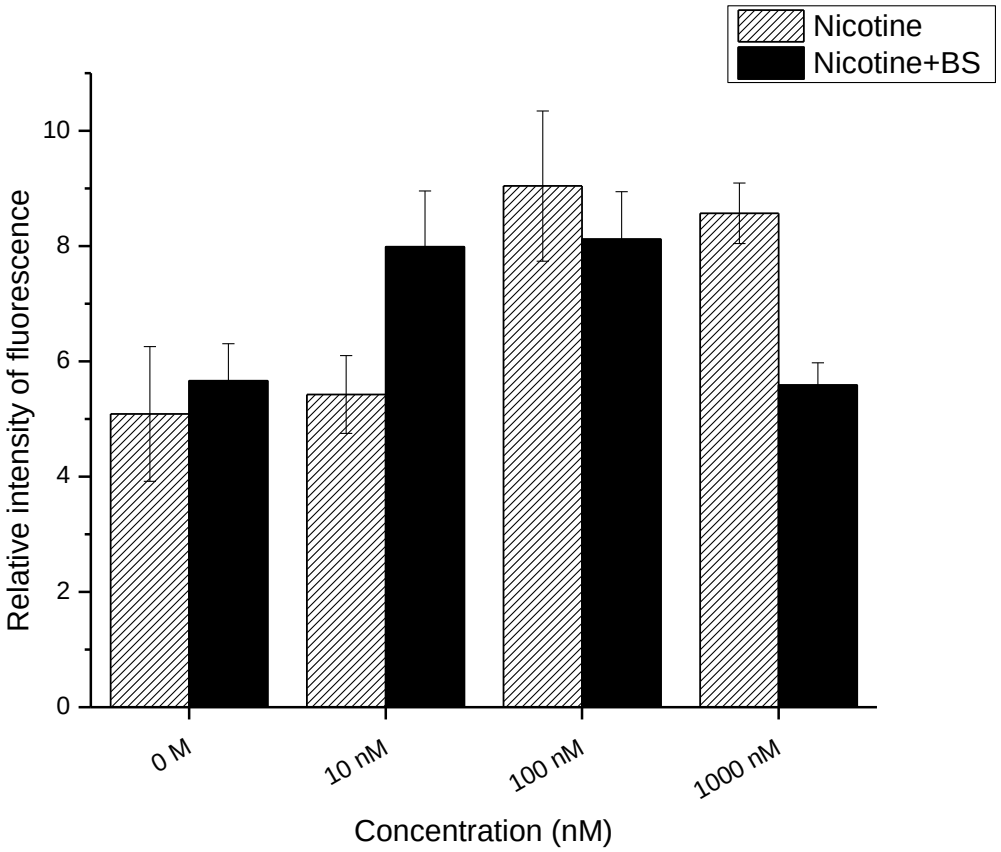


Figure 3.16- Schematic showing the relative fluorescence intensity of cells exposed to nicotine and ICCM. The intensity increased when nicotine cells were exposed to nicotine with a threshold of 100 nM. When ICCM was added this increase shifted to 10 nM and decreased for 1000 nM

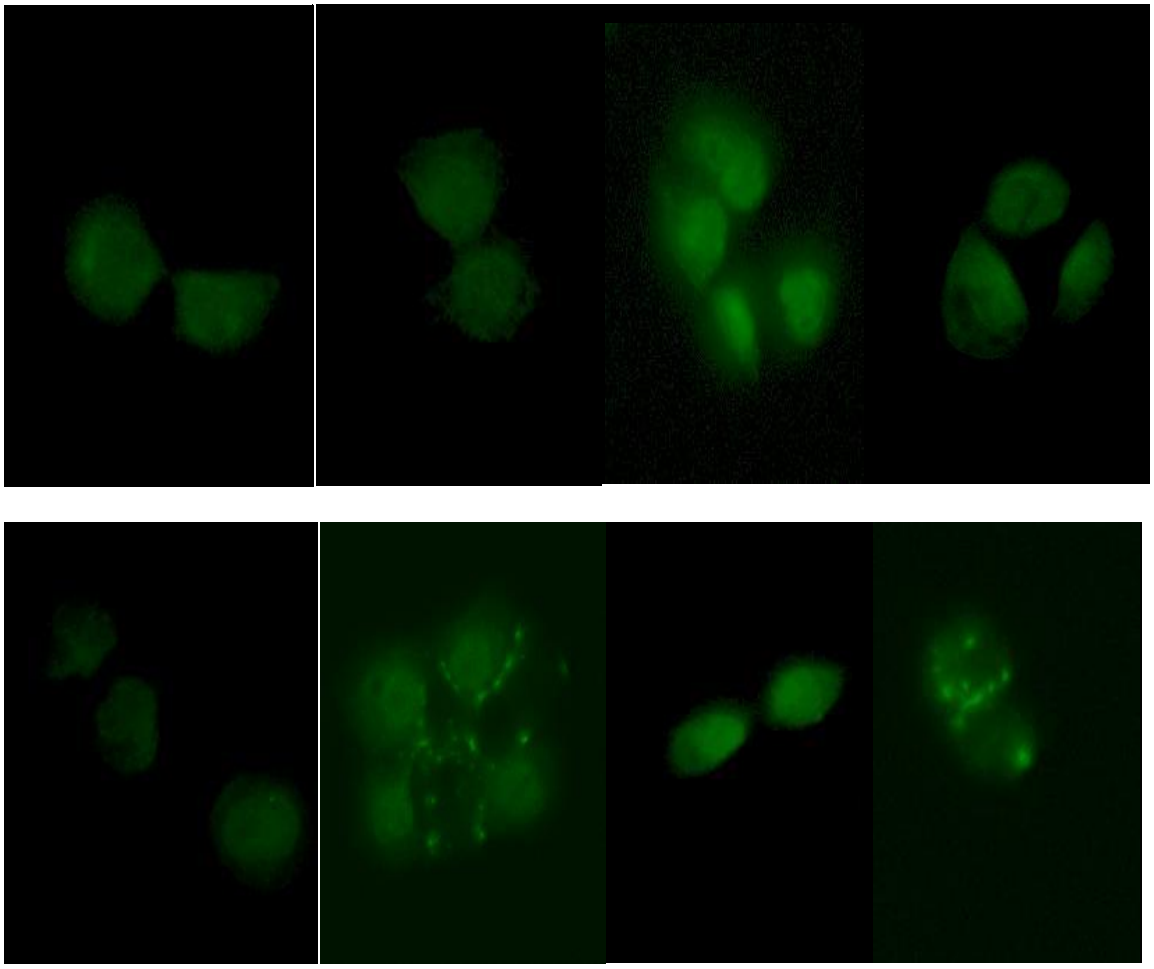


Figure 3.17- Illustration of immunofluorescence images of HPV-G cells. Top: cells exposed to nicotine concentration (0 M, 10 nM, 100 nM, and 1000 nM). Bottom: cells exposed to ICCM and nicotine (0 M, 10 nM, 100 nM, and 1000 nM)

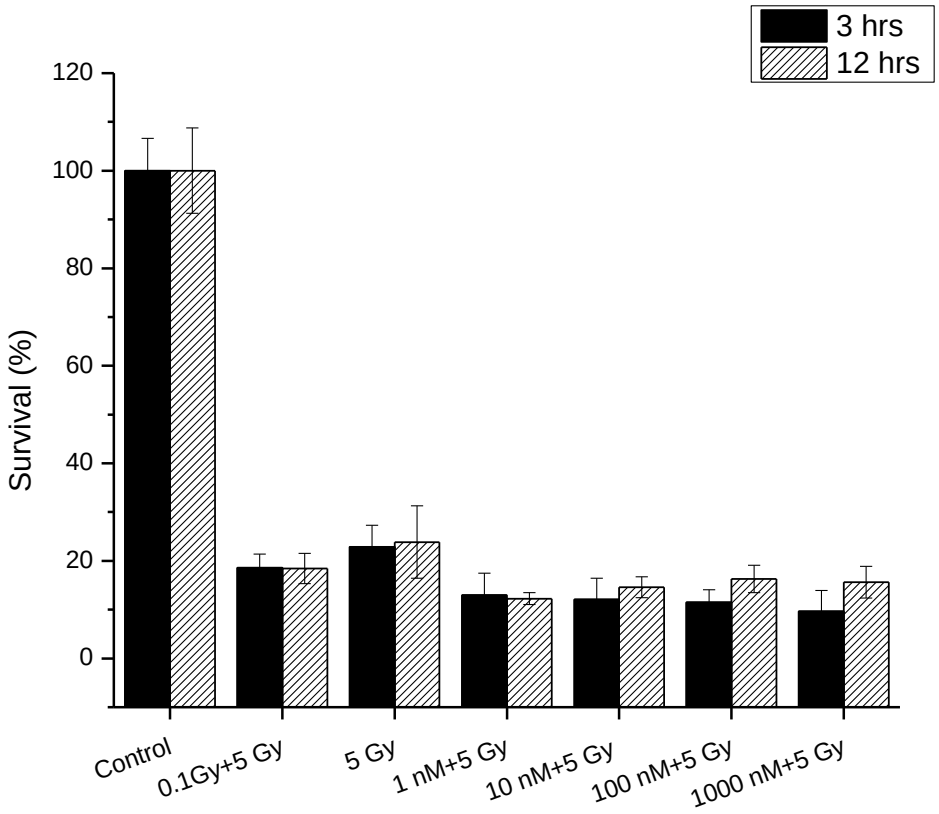


Figure 3.18- Radiation and nicotine induced adaptive response of HPV-G cells. It was observed that increasing the time between priming and challenge stress had no effect on the survival of the cells. A slight decrease in survival was observed in cells exposed primarily to 100 mGy or nicotine when compared to the survival of the cells which received 5 Gy only and no priming stress

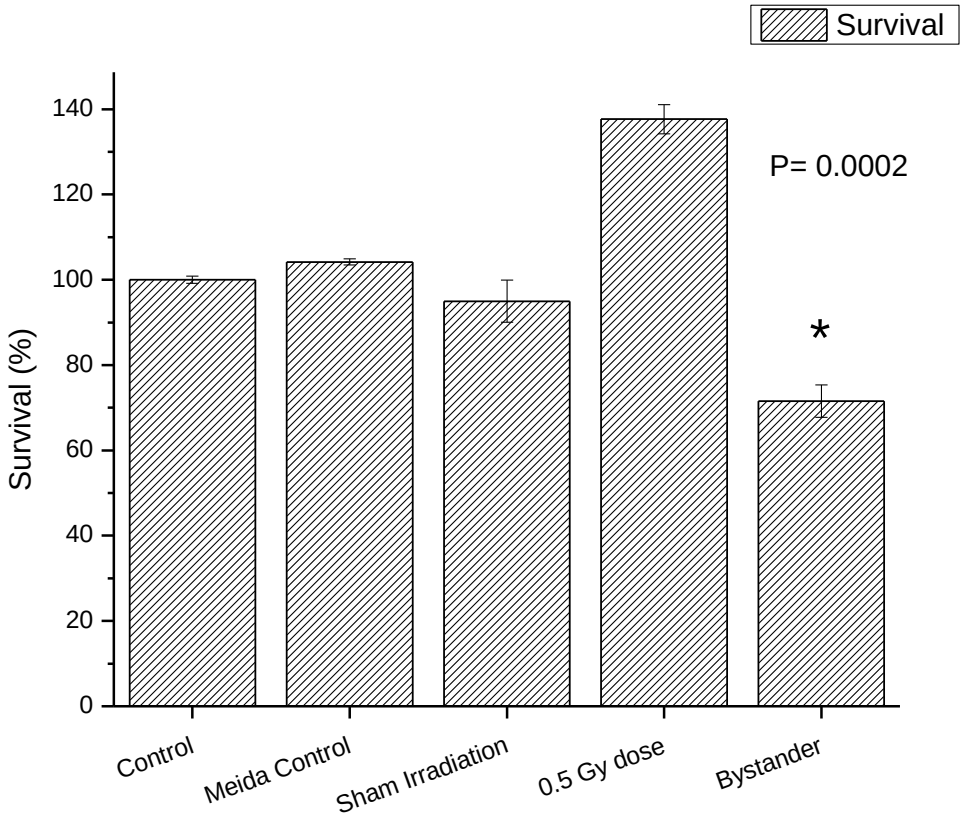


Figure 3.19- Radiation-induced bystander effect on RT-112 cell. The bystander group showed a significantly lower survival compared to the controls. Interestingly, 0.5 Gy gammas enhanced the survival of this human bladder carcinoma cell line

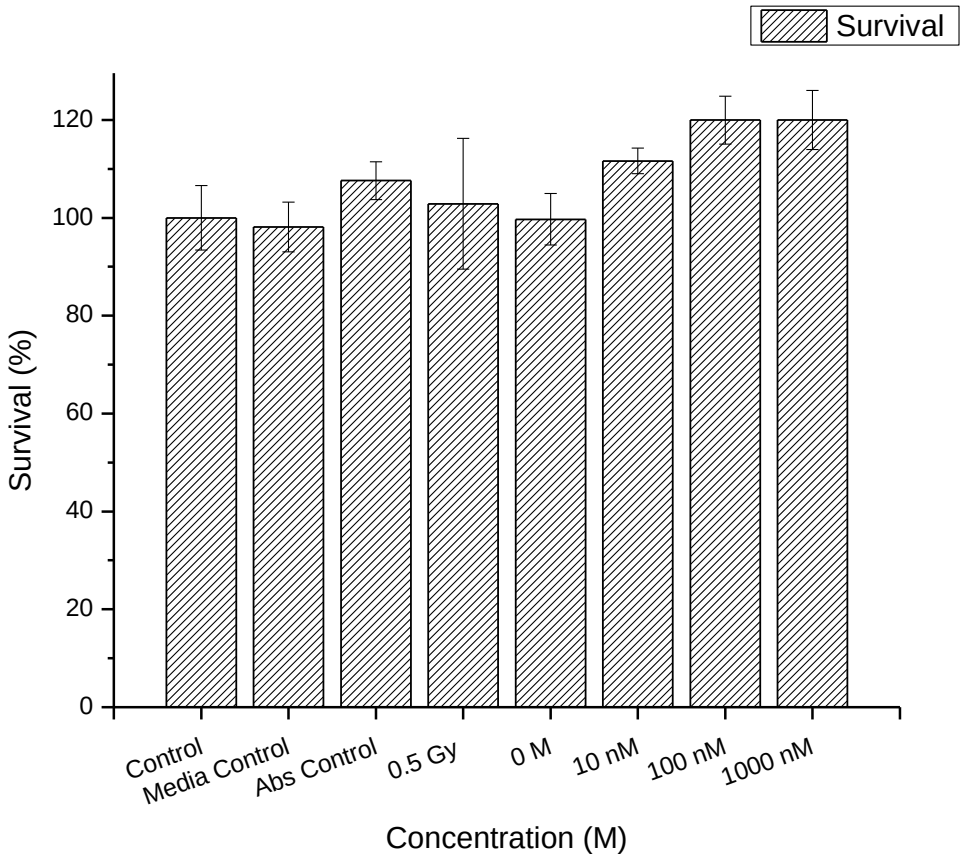


Figure 3.20- Effect of nicotine on radiation-induced bystander response of RT-112 cells. The results showed that survival of the cells increased with the increasing concentration of nicotine from 10 nM to 1000 nM

Table 3.1- Survival, error, and P values of the kinetic experiment showing the significant changes

Concentration (M)	Time (Hours)	Mean Survival (%)		Standard Error of the Mean (SEM)		P Value
		Control	0.5 Gy	Control	0.5 Gy	
10 nM	2h	115.5	112.4	4.6	11.3	0.88
	6h	106.8	98.2	3.9	2.4	0.045
	24h	135.8	118.3	4.9	2.2	0.05
	240	83.5	65.6	5.9	3.2	0.03
100 nM	2h	119.9	2.2	2.7	114.7	0.21
	6h	125.8	6.5	3.8	128.9	0.70
	24h	120.2	5.9	6.3	115.2	0.59
	240	58.3	3.8	16.4	59.4	0.95
1000 nM	2h	134.7	113.8	5.3	7.1	0.07
	6h	125.8	99.6	2.2	2.9	0.002
	24h	150.3	126.4	13.7	6.8	0.19
	240	54.7	52.2	12.8	7.7	0.87

Table 3.2- Survival, error, and P values for 0.1 Gy and 0.5 Gy doses for varying concentrations of nicotine. For concentrations of 100 nM and beyond there is a significant reduction in the survival of the cells exposed to 0.5 Gy

Concentration (M)	Mean Survival (%)		Standard Error of the Mean (SEM)		P Value
	0.1 Gy	0.5 Gy	0.1 Gy	0.5 Gy	
0 M	97.3	97.6	3.2	5.5	0.96
0.1 nM	106.0	80.7	5.1	6.5	0.03
1 nM	99.5	95.2	6.8	10.1	0.74
10 nM	88.5	80.1	1.9	4.8	0.17
100 nM	89.2	72.2	4.5	4.9	0.06
1 µM	99.2	78.5	3.5	6.5	0.04
10 µM	96.5	72.8	3.9	2.13	0.005
100 µM	70.9	66.7	0.4	0.4	0.001

CHAPTER 4

DISCUSSION

It has been more than 100 years since the initial discovery of nicotinic receptors. An extensive and valuable body of research can be found on the effect nicotine on nAChRs. However, the underlying mechanisms are still to be elucidated as the effects mediated through exposure to nicotine can depend on a variety of factors such as the model and cell line used, the endpoints studied, drug concentration, and also duration of exposure. The same applies to radiation-induced bystander effects and in general to non-targeted effects of ionizing radiation. The pathways through which these signals influence the cells are also under investigation. Studying the effects of two such effects in conjunction presents even more complications in terms of interpretation of the results observed and investigation of their mechanisms, but as the reality of life is that people are seldom exposed to single stressors and smoking is a confounding factor in radiotherapy and other medical radiation exposure studies, it is important to study the interactions. The present chapter attempts to interpret some of the results achieved during the course of the research through finding correlation between different endpoints and also previously published data in this area.

4. 1- Kinetics of Nicotine in HPV-G Cells

In this experiment a distinct kinetic behaviour was observed after cells were treated with different nicotine concentrations and gamma radiation. There was an insignificant decrease in survival from 2 to 6 hours for 10 and 1000 nM followed by an increase that continued until 24 hours after exposure to nicotine. This was then followed by a remarkable decrease for the rest of the incubation time i.e. 240 hours. The increased growth during the short-term exposure further confirmed the results of a 1993 study by Grando *et al* who showed that short-term administration of nicotine causes enhanced cytoplasm motility and lateral migration of the cells and therefore, facilitated a stronger cell-cell and cell-substrate adhesion in keratinocytes [116,117]. The gradual drop in survival from 24 to 240 hours can be attributed to shrinkage and detachment of cells which was reported in keratinocytes by Zia *et al*,1997 [118] and also cytotoxic effect of nicotine, these effects are often seen when cells are reposed to drugs for a long time [119]. The response of the cells during 2 to 6 hours post incubation with nicotine varied greatly with respect to concentration and time. At 2 hours the survival for all concentrations was similar with an increase of about 10% relative to the controls which failed to reach significance. From 2 to 6 hours this behaviour turned into a bimodal concentration-dependent manner where the survival of the cells increased significantly after administration of 100 nM of nicotine compared to 10 and 1000 nM which may indicate that there exists an optimal concentration that enhances cell proliferation in shorter exposure times (Figure 3.1). When irradiated to 0.5 Gy, the same pattern of

response was observed, however, the overall survival underwent a decrease due to cell killing effects of radiation (Figure 3.2). Figures 3.3 to 3.5 depict and further confirm the detrimental effects of radiation on cell survival where a marked decrease was observed comparing to the control group. Interestingly, radiation did not seem to affect the survival at 100 nM, indicating that effects mediated at this concentration were more influential than exposure to ionizing radiation.

4. 2- Prolonged Exposure of HPV-G Cells to Nicotine

The relevance of prolonged exposure studied in the present thesis lies in the continued exposure of a smoker to nicotine due to regular smoking sessions which prevents the clearance of nicotine even during overnight abstention. Shown in Figure 3.6, is a bimodal response that, except for 1 μM, increases to reach a significant maximum at 10 μM and falls down as concentration extends to 1 mM, the concentration shown repeatedly as the toxic concentration to the cell lines used in this study. Lower concentrations however did not seem to have an effect on survival of the cells as tested by clonogenic assay. The bimodal behaviour of nicotine was also observed by Villablanca *et al* in 1998, Walker in 2001, and Poon *et al* in 2007 [30,31,120]. Villablanca's team reported a nicotine induced proliferation in calf endothelial cells for concentrations below 10^{-8} M and cytotoxicity in concentrations of 10^{-6} M and higher and suggested possible implications in tumor angiogenesis; this was shown to be a result of nicotine-induced DNA synthesis in these cells [31]. Walker showed that 0.01-10 μM of

nicotine increased the proliferation of osteosarcoma cells while concentrations as high as 10-10,000 μM hampered the effect, leading to cell death. The result of the current experiment is in agreement with the aforementioned studies in that a bimodal response is observed; however, the range for this response is quite different. This may be explained through variations in different studies in terms of the cell lines and the incubation times of a given study as well as the endpoints chosen. Nicotine is known to confer resistance against apoptosis in various cell lines, both *in vivo* and *in vitro*, which is proposed as one of the possible pathways for nicotine-induced survival [22]. Therefore, the increase observed in the results can be explained through anti-apoptotic effects of nicotine and the decrease can be attributed to the dose-dependent increase in cytotoxicity caused by nicotine.

4. 3- Effect of the Sequence of Exposure to Nicotine and Radiation

The dose-response curves depicted in Figure 3.7 clearly indicate that the sequence of exposure to nicotine and radiation influences the outcome in terms of cell survival. In both cases (i.e. irradiation before and after exposure to nicotine) the survival dropped below the control level, however, incubation with nicotine for 18 hours before irradiation conferred resistance against radiation-induced cell killing. This effect did not appear to be concentration-sensitive in the nano-molar ranges. A similar response was reported by Mosse *et al* in 2006 when melanin was added to HPV-G cells where adding melanin before irradiation rendered a protective behaviour [72]. The observed phenomenon seemed to correlate, once again, with anti-apoptotic properties of nicotine. This may have

important implications in radiotherapy, as nicotine-induced protection against cell death in tumor cells would mean less probability for an effective treatment in smokers. The same anti-apoptotic response was reported by Wright *et al* in 1993 against chemotherapeutic drugs, leading them to suggest that nicotine may promote new tumors to emerge [35]. A more detailed look at the results of post-irradiation nicotine exposure further showed a concentration-dependent increase in survival at high doses, more specifically 5 Gy, which could suggest a weak compensatory response induced by nicotine; this effect, however, may be insignificant in lower doses and more pronounced in higher doses.

4. 4- Nicotine and Radiation-induced Bystander Effect

Radiation-induced bystander signals have been the motivation for many studies and therefore, well studied and established [59,61,73,121–123]. Extensive efforts have been made to elucidate the underlying mechanisms of this effect and its implications in health risks and cancer treatment. However, very little research has been done concerning exposure of the public to both. The extent of research done on this subject is limited to the work done by Poon *et al* 2007 where an elevated survival was observed at 100 nM in a preliminary proof of principle experiment [30]. Radiation-induced bystander signal has been shown to induce calcium influx in the recipient cells as the first response to addition of ICCM [30,124,125]. Interestingly, this effect was removed when different voltage-dependent calcium channel blockers were administered; indicating that the calcium influx

pathway is mostly through these channels. This effect was only partially reduced when Thapsigargin, which depletes the intracellular calcium stores, was added; suggesting that intracellular calcium stores also have a role in the nature of this calcium influx. Additionally, this was shown to be followed by apoptosis in the cells as the predominant mode of cell death in RIBE [23]. Nicotine, on the hand has shown to exhibit proliferative and anti-apoptotic effects in keratinocytes [5,21]. Moreover, the role of nicotine in inducing calcium influx through activation of nAChRs is widely accepted, as these receptors are permeable to influx of Ca^{2+} and Na^{+} and efflux of K^{+} [126]. Furthermore, calcium release from intracellular stores is considered a pathway for more calcium influx downstream of nAChR activation (especially α7) [127]. Keratinocytes express α (α3, α5, and α7) and β (β2 and β4) nicotinic subunits [32,117], however, only α subtypes are associated with agonist binding. In oral keratinocytes, prolonged exposure to nicotine causes the composition of the subunits to change from α3 to α7 [38]. Among the subunits, α7 has the greatest calcium permeability and is associated with cell differentiation [38,127]. These receptor alterations may be the reason behind the differences between acute and chronic exposure to nicotine and nicotine cytotoxicity [32]. The results obtained in this research clearly demonstrated two separate patterns for low doses and high doses with a cut-off point of 1 Gy. Two distinct phenomena were observed in Figure 3.8. The bimodal nature of the response can be attributed to the effects of nicotine as it has been shown in the previous sections. There was however, a decrease in survival in the low-dose range compared to previous results of prolonged exposure to nicotine observed which may be the result of ICCM-induced cell death. The decreased survival together

with the shift in the bimodal response towards the lower concentration leads to the suggestion that there may be a delicate balance between the two effects. One possibility may be the differences due to downstream pathways from different calcium influx sources i.e. voltage-dependent calcium channels and permeability through nAChRs. The mechanisms behind the dose-dependent bimodal response to nicotine, however, are not yet understood, though receptor desensitization could be a potential possibility which requires further research. The differences from low doses to high doses were subtle and resulted predominantly from the increased survival observed at 100 μM. The general pattern for survival with respect to different doses was a decrease up to 1 Gy followed by an increase most dominantly at 5 Gy. This is in agreement with the previously published data indicating that increasing the dose does not result in an elevated cell death [128]. It appears that nicotine's proliferative effects overcome cell death in the presence of a saturated bystander signal. Same principles may be applied to the results depicted in Figure 3.11 where a similar pattern was observed for low vs. high concentrations of nicotine confirming a bimodal nature.

As already discussed, there are considerable differences between acute and prolonged effects of nicotine exposure. This was investigated in the context of RIBE, shown in Figure 3.12. The clear, significant decrease in survival observed with no effect on the bimodal nature of nicotine suggests a role for receptor composition changes as well as ICCM-induced cell killing. As mentioned before, long exposure to nicotine triggers a change in receptor number and composition in favour of α7 subunit which has a established role in cell proliferation and differentiation. An acute exposure, which is

essentially shorter than the clearance half life of nicotine, may not lead to such proliferative results. This may in turn leave room for apoptotic cell death induced by ICCM, which was previously suppressed by the dominating effects on nicotine, resulting in a decreased survival rate in comparison to chronic exposure.

Another interesting point observed was the effect of different serums on the radiation-induced bystander response of cells exposed to nicotine. The results depicted in Figures 3.13 and 3.14 show great variations in survival for different batches of serum. This was in complete agreement with the previous studies where different levels of serotonin proved to have a crucial importance in the radiation-induced bystander effects [30,84]. Therefore, the observed changes in survival may be ascribed to the differences in serotonin level in these serums, but this cannot be confirmed without a detailed analysis of the batches used.

4. 5- Viability assay

Viability assays have been used in the literature for the assessment of both nicotine effects and RIBE. Lyng *et al* used Alamar blue to investigate the viability of cells exposed to ICCM and different inhibitors of RIBE [128]. The 2005 work of Lee *et al* using MTT on various cell lines showed a cell line dependent decrease in viability after 5 days of incubation with no change in viability for HN12 metastatic oral cancer cell line [129]. Gray and Clothier reported an increase in metabolic activity of human primary keratinocytes using Alamar blue over 6 and 12 days of incubation, the effect diminished

at high concentrations [130]. The results of viability assay in the present research confirm the work of Gray and Clothier. Viability results are also in perfect agreement with the findings of clonogenic assay discussed in the previous section. Both endpoints confirmed a fall in viability and survival for nano-molar ranges followed by a rise at 1 μM and a subsequent drop at 10 μM. At 5 Gy the same results were observed with the minimum and maximum viability and survival happening at 10 nM and 1 μM, respectively (Figure 3.15).

4. 6- Immunofluorescence Assay

Immunofluorescence is a valuable tool in receptor visualization, especially in characterization of different nAChR subtypes and their expression after a certain treatment [131–133]. The antibody used in this study visualizes α1, α3, and α5 subtypes. It was chosen because it covers more subunits than the other available alternative which was specifically designed for α7 subunit. Moreover, the effect of α7 is more evident in chronic incubations whereas the incubation time of the present experiment was only one hour in order to keep the experiments consistent with the general bystander protocol. The absence of α7 subunit prevents a thorough comparison between the results of the immunofluorescence intensity and survival of the nicotine bystander experiments. Therefore, a comparison is made with results of short-term exposure to nicotine shown in Figure 3.12 where the effect of α7 subunit, however still present, is less influential. Presence of nicotine at concentrations of 100 nM and 1 μM increased the intensity of

fluorescence indicating an increase in the expression of the receptor which is a generally accepted behaviour of the receptor in response to nicotine [32,134]. Exposure to both nicotine and ICCM for one hour shifted the intensity levels to lower concentrations consistent with the result of clonogenic survival after one hour exposure (Figure 3.12). Over expression of the receptors upon administering ICCM was only observed at 10 nM, after which this over expression experienced a drop in intensity in the absence of nicotine, indicating that increased expression of the nAChRs after short exposures at higher concentrations may be hampered by the radiation-induced bystander effects.

4. 7- Adaptive response

Radiation-induced adaptive responses have been subject to intensive investigations [75,88,100,102,108,123,135]. Such responses, however, are not observed in all cell types. Given the fact that nicotine is present in various concentrations in smokers, the possibility of rendering a protective effect due to proliferative properties of nicotine was of interest. HPV-G cells did not exhibit an adaptive response as confirmed in the work of Ryan *et al* [75]. No protective response was observed when 0.1 Gy gammas or nicotine concentrations where administered as a priming stress. To ensure that the lack of adaptive response was not due to the time gap of 12 hours between the priming dose and challenge dose, a second experiment was designed with a time gap of 3 hours. No adaptive response was exhibited by the HPV-G cells. Survival was lower when nicotine was used as the priming dose compared to 0.1 Gy gammas after 3 hours,

indicating that nicotine is not a suitable priming stressor for adaptive responses. The fact that a change in time gap rendered no significant difference in survival suggests that even though exposure to nicotine has shown to stimulate DNA synthesis [31], pathways through which nicotine functions offer no protection against the DNA damage caused by radiation (Figure 3.18).

4. 8- Nicotine and Radiation-induced Bystander Effect in RT-112 Cells

As mentioned before, not all cell lines can produce bystander signal nor can all respond to the generated signal, confirming cell line-specific nature of the phenomenon [76,94]. This research demonstrated the radiation-induced bystander effect in RT-112 cell line for the first time, confirming that this cell line is capable of both producing the signal and responding to it (Figure 3.19).

The response to nicotine exposure and the demonstrated lack of loss in survival can be an evidence for a more dominant nicotinic receptor response affecting or saturating the bystander signal clearly produced in the cells. α7 is the most highly expressed subtype in human bladder urothelium, followed by α5 and α3 [136,137]. The dominant presence of α7 and the proliferative and differentiation-inducing effects associated with it may be the underlying reason for an increased survival in range of concentrations studied.

4.9- Conclusions

To conclude, this study sheds some light on the impact of nicotine on low dose and non-targeted effects of ionizing radiation. The kinetic study revealed the bimodal nature of nicotine effect on HPV-G cells as well as the detrimental effect of low doses of radiation on the proliferation induced by nicotine. Prolonged exposure to nicotine further confirmed the bimodal effects of nicotine and set a base line for comparing the rest of the experiments. Pre-incubation with nicotine seemed to confer resistance against a subsequent insult, i.e. radiation, whereas exposure to nicotine did not result in the same protective effects. Radiation-induced bystander signal together with nicotine showed interesting results where both bimodal effects of nicotine and bystander-induced cell killing could be observed simultaneously. These results were further confirmed through viability assay and immunofluorescence visualization of receptors. HPV-G cells did not show an adaptive response to neither radiation nor nicotine exposure as priming stress under the experimental design of this research. RT-112 cell line was shown to be an interesting candidate for further bystander experiment, as well as a suitable cell line for nicotine experiments considering the receptors subuints and its response to nicotine bystander signal.

4.10- Future Directions

It would be interesting to look in more detail at the underlying mechanisms and pathways through which the observed responses function. Investigation of calcium influx in HPV-G cells following exposure to nicotine and fully characterizing the source of this calcium and duration of its presence and magnitude of the nicotine-induced calcium signal compared to its ICCM counterpart may shed more light on the nature of the responses. Combination of apoptotic effects of ICCM and anti-apoptotic properties of nicotine sets the scene for exciting research on the interaction between these two phenomena. Moreover, using more specific antibodies and monitoring the effects in different time frames may reveal interesting information about the changes in receptor composition following nicotine exposure in these cells and whether exposure to ICCM and the calcium influx associated with it has an effect on the composition changes. As far as the adaptive responses are concerned, studying other relevant cell lines and also acute exposures to nicotine as a therapeutic approach may lead to worthy results.

Also, extending this research to *in vivo* studies with a suitable model can yield valuable data on how nicotine and radiation interact in living models. Additionally, tobacco smoke, being comprised of various carcinogens may provide a more realistic view on the interaction of different stressors and carcinogens.

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APPENDIX

Raw Data

Kinetics of nicotine

0.5 GY	10nM							
	count	count	count	C/PE	C/PE	C/PE	AVE	SEM
2h	117	125	161	97.9	104.6	134.7	112.4	11.3
6h	114	115	123	95.4	96.2	102.9	98.2	2.4
24h	144	144	136	120.5	120.5	113.8	118.3	2.2
240	75	86	74	62.8	71.9	61.9	65.6	3.2
0.5 GY	100nM							
	count	count	count	C/PE	C/PE	C/PE	AVE	SEM
2	133	142	136	111.3	118.8	113.8	114.6	2.2
6	168	141	153	140.6	117.9	128.0	128.9	6.5
24	124	148	141	103.8	123.8	117.9	115.2	5.9
240	67	66	80	56.1	55.2	66.9	59.4	3.7
0.5 GY	1000nM							
	count	count	count	C/PE	C/PE	C/PE	AVE	SEM
2h	123	133	152	102.9	111.3	127.2	113.8	7.1
6h	119	113	125	99.6	94.6	104.6	99.6	2.9
24h	145	141	167	121.3	117.9	139.7	126.4	6.8
240	80	58	49	66.9	48.5	41.0	52.2	7.7

0 GY	**10 nM**							
	count	**count**	**count**	**C/PE**	**C/PE**	**C/PE**	**AVE**	**SEM**
2h	144	127	143	120.5	106.3	119.7	115.5	4.6
6h	122	137	124	102.1	114.6	103.8	106.8	3.9
24h	151	171	165	126.4	143.1	138.1	135.8	4.9
240	86	105	109	71.9	87.9	91.2	83.7	5.9
0 GY	**100 nM**							
	count	**count**	**count**	**C/PE**	**C/PE**	**C/PE**	**AVE**	**SEM**
2h	137	145	148	114.6	121.3	123.8	119.9	2.7
6h	144	148	159	120.5	123.8	133.1	125.8	3.8
24h	156	145	130	130.5	121.3	108.8	120.2	6.3
240	58	43	108	48.5	35.9	90.4	58.3	16.4
0 GY	**1000 nM**							
	count	**count**	**count**	**C/PE**	**C/PE**	**C/PE**	**AVE**	**SEM**
2h	150	161	172	125.5	134.7	143.9	134.7	5.3
6h	155	150	146	129.7	125.5	122.1	125.8	2.2
24h	211	172	156	176.6	143.9	130.5	150.3	13.7
240	94	42	60	78.7	35.1	50.2	54.8	12.8

Prolonged Exposure to Nicotine

	Count	Count	Count	Survival	Survival	Survival	Mean Survival	SEM
Control	67	78	88	86.2	100.4	113.3	100	7.8
0.1 nM	47	75	76	60.5	96.6	97.9	84.9	12.2
1 nM	57	62	80	73.4	79.8	103.0	85.4	8.9
10 nM	64	55	48	82.4	70.8	61.8	71.7	5.9
100 nM	80	82	61	103.0	105.6	78.5	95.7	8.6
1 µM	66	70	70	84.9	90.1	90.1	88.4	1.7
10 µM	101	80	83	130.0	103.0	106.9	113.3	8.4
100 µM	64	64	61	82.4	82.4	78.5	81.1	1.3
1mM	0	0	0	0	0	0	0	0

Sequence of Exposure to Nicotine and ICCM

	Before	Before	Before	Before	Before	Before	Before	Before
0 nM	**Count**	**Count**	**Count**	**Survival**	**Survival**	**Survival**	**Mean Survival**	**SEM**
Control	163	164	162	100	100.6	99.4	100	0.4
0 Gy	163	164	162	100	100.6	99.4	100	0.4
0.1 Gy	185	188	174	113.4969	115.3	106.7	111.9	2.6
0.5 Gy	189	162	148	115.9509	99.4	90.8	102.05	7.4
1 Gy	161	149	138	98.77301	91.4	84.7	91.6	4.1
2 Gy	158	139	127	96.93252	85.3	77.9	86.7	5.5
3 Gy	189	199	162	57.97546	61.0	49.7	56.2	3.4
5 Gy	329	343	363	20.18405	21.0	22.3	21.2	0.6

	Before	Before	Before	Before	Before	Before	Before	Before
1 nM	**Count**	**Count**	**Count**	**Survival**	**Survival**	**Survival**	**Mean Survival**	**SEM**
Control	163	164	162	100	100.6	99.4	100	0.4
0 Gy	167	165	194	102.5	101.2	119.0	107.7	5.7
0.1 Gy	139	164	147	85.3	100.6	90.2	92.0	4.5
0.5 Gy	163	114	134	100	69.9	82.2	84.0	8.7
1 Gy	82	109	118	50.3=	66.9	72.4	63.2	6.6
2 Gy	125	131	139	76.7	80.4	85.3	80.8	2.5
3 Gy	86	135	132	26.4	41.4	40.5	36.1	4.9
5 Gy	384	363	277	23.6	22.3	16.9	20.9	2.0

	Before	Before	Before	Before	Before	Before	Before	Before
10 nM	**Count**	**Count**	**Count**	**Survival**	**Survival**	**Survival**	**Mean Survival**	**SEM**
Control	163	164	162	100	100.6	99.4	100	0.4
0 Gy	151	190	194	92.6	116.6	119.0	109.4	8.4
0.1 Gy	155	143	182	95.1	87.7	111.7	98.2	7.1
0.5 Gy	134	157	165	82.2	96.3	101.2	93.3	5.7
1 Gy	151	174	108	92.6	106.7	66.3	88.5	11.9
2 Gy	121	152	174	74.2	93.3	106.7	91.4	9.4
3 Gy	147	147	94	45.1	45.1	28.9	39.7	5.4
5 Gy	300	326	363	18.4	20	22.3	20.2	1.1

	Before	Before	Before	Before	Before	Before	Before	Before
100 nM	**Count**	**Count**	**Count**	**Survival**	**Survival**	**Survival**	**Mean Survival**	**SEM**
Control	163	164	162	100	100.6	99.4	100	0.6
0 Gy	190	174	191	116.6	106.7	117.2	113.5	3.4
0.1 Gy	189	179	162	115.9	109.8	99.4	108.4	4.8
0.5 Gy	142	152	170	87.1	93.3	104.3	94.9	5.0
1 Gy	188	122	105	115.3	74.8	64.4	84.9	15.5
2 Gy	74	71	105	45.4	43.6	64.4	51.1	6.7
3 Gy	202	171	184	61.9	52.5	56.4	56.9	2.8
5 Gy	325	346	246	19.9	21.2	15.1	18.8	1.9

	After	After	After	After	After	After	After	After
0 nM	**Count**	**Count**	**Count**	**Survival**	**Survival**	**Survival**	**Mean Survival**	**SEM**
Control	178	203	198	92.2	105.2	102.6	100	3.9
0 Gy	178	203	198	92.2	105.2	102.6	100	3.9
0.1 Gy	105	94	84	54.4	48.7	43.5	48.9	3.1
0.5 Gy	102	115	75	52.8	59.6	38.9	50.4	6.1
1 Gy	103	120	70	53.4	62.2	36.3	50.6	7.6
2 Gy	138	69	115	71.5	35.8	59.6	55.6	10.5
3 Gy	143	191	34	37.0	49.5	8.8	31.8	12.0
5 Gy	23	22	10	1.2	1.1	0.5	0.9	0.2

	After	After	After	After	After	After	After	After
1 nM	**Count**	**Count**	**Count**	**Survival**	**Survival**	**Survival**	**Mean Survival**	**SEM**
Control	178	203	198	92.2	105.1	102.6	100	3.9
0 Gy	167	165	194	86.5	85.5	100.5	90.8	4.8
0.1 Gy	112	132	225	58.0	68.4	116.6	81.0	18.0
0.5 Gy	152	205	63	78.8	106.2	32.6	72.5	21.5
1 Gy	67	42	110	34.7	21.8	56.9	37.8	10.3
2 Gy	21	24	91	10.9	12.4	47.2	23.5	11.9
3 Gy	45	35	78	11.7	9.1	20.2	13.6	3.4
5 Gy	5	1	4	0.3	0.1	0.2	0.2	0.1

	After	After	After	After	After	After	After	After
10 nM	**Count**	**Count**	**Count**	**Survival**	**Survival**	**Survival**	**Mean Survival**	**SEM**
Control	178	203	198	92.2	105.2	102.6	100	3.9
0 Gy	88	246	186	45.6	127.5	96.4	89.8	23.9
0.1 Gy	41	71	69	21.2	36.8	35.8	31.3	5.0
0.5 Gy	63	139	170	32.6	72.0	88.1	64.2	16.5
1 Gy	37	34	18	19.2	17.6	9.3	15.4	3.1
2 Gy	34	38	62	17.6	19.7	32.1	23.14335	4.5
3 Gy	95	88	48	24.6	22.8	12.4	19.9	3.8
5 Gy	36	2	2	1.9	0.1	0.1	0.7	0.6

	After	After	After	After	After	After	After	After
100 nM	**Count**	**Count**	**Count**	**Survival**	**Survival**	**Survival**	**Mean Survival**	**SEM**
Control	178	203	198	92.2	105.2	1026	100	3.9
0 Gy	120	63	226	62.2	32.69	117.1	70.6	24.7
0.1 Gy	57	85	106	29.5	44.0	54.9	42.8	7.4
0.5 Gy	101	89	80	52.3	46.1	41.5	46.6	3.1
1 Gy	50	68	41	25.9	35.2	21.2	27.5	4.1
2 Gy	177	97	45	91.7	50.3	23.3	55.1	19.9
3 Gy	87	86	79	22.5	22.3	20.5	21.8	0.7
5 Gy	356	6	5	18.4	0.3	0.3	6.3	6.1

Nicotine and Radiation-induced Bystander Effect

0.1 Gy								
	Count	**Count**	**Count**	**Survival**	**Survival**	**Survival**	**Mean Survival**	**SEM**
Control	225	196	208	107.3	93.1	99.2	100	4.0
Media	208	235	222	99.2	112.1	105.9	105.7	3.7
Abs control	196	193	228	93.5	92.1	108.7	98.1	5.3
0.5 Gy	225	215	227	107.3	102.5	108.3	106.0	1.7
0 M	201	217	194	95.95	103.5	92.5	97.3	3.2
0.1 nM	235	231	201	112.1	110.2	95.9	106.0	5.1
1 nM	188	236	202	89.7	112.6	96.3	99.5	6.8
10 nM	194	181	182	92.5	86.3	86.8	88.5	1.9
100 nM	170	188	203	81.1	89.7	96.8	89.2	4.5
1 µM	211	219	194	100.6	104.4	92.5	99.2	3.5
10 µM	201	217	189	95.9	103.5	90.1	96.5	3.9
100 µM	295	301	297	70.3	71.8	70.8	70.9	0.4
1 mM	0	0	0	0	0	0	0	0

0.5 Gy								
	Count	Count	Count	Survival	Survival	Survival	Mean Survival	SEM
Control	212	222	235	95.1	99.6	105.4	100	2.9
Media	209	179	168	93.7	80.3	75.3	83.1	5.5
Abs control	202	222	160	90.6	99.6	71.7	87.3	8.2
0.5 Gy	122	148	144	54.7	66.3	64.6	61.9	3.6
0 M	230	230	193	103.2	103.1	86.5	97.6	5.5
0.1 nM	209	163	168	93.7	73.1	75.3	80.7	6.5
1 nM	215	250	172	96.4	112.1	77.1	95.2	10.1
10 nM	200	169	167	89.7	75.8	74.9	80.1	4.8
100 nM	151	149	183	67.7	66.8	82.1	72.1	4.9
1 µM	163	204	158	73.1	91.5	70.92	78.54	6.5
10 µM	168	166	153	75.3	74.4	68.6	72.8	2.1
100 µM	295	301	297	66.1	67.4	66.6	66.7	0.4
1 mM	0	0	0	0	0	0	0	0

1 Gy								
	Count	Count	Count	Survival	Survival	Survival	Mean Survival	SEM
Control	213	204	237	97.7	93.5	108.7	100	4.5
Media	240	261	262	90.5	98.4	98.8	95.9	2.7
Abs control	172	156	145	78.8	71.5	66.5	72.3	3.6
0.5 Gy	192	267	200	88.0	122.4	91.7	100.7	10.9
0 M	149	152	152	68.3	69.7	69.7	69.2	0.4
0.1 nM	130	188	144	59.6	86.2	66.1	70.6	8.0
1 nM	171	222	177	78.4	101.8	81.1	87.1	7.3
10 nM	144	130	148	66.0	59.6	67.8	64.5	2.5
100 nM	141	150	78	64.6	68.8	35.7	56.4	10.3
1 µM	194	155	162	88.9	71.1	74.3	78.1	5.5
10 µM	154	147	150	70.6	67.4	68.8	68.9	0.9
100 µM	493	408	495	92.6	76.6	93.0	87.4	5.3
1 mM	0	0	0	0	0	0	0	0

2 Gy								
	Count	Count	Count	Survival	Survival	Survival	Mean Survival	SEM
Control	182	185	161	103.4	105.1	91.4	100	4.2
Media	137	127	123	77.8	72.1	69.8	73.2	2.3
Abs control	158	186	173	89.7	105.6	98.2	97.9	4.5
0.5 Gy	132	79	95	75	44.8	53.9	57.9	8.9
0 M	161	176	230	91.4	100	130.6	107.3	11.9
0.1 nM	142	112	133	80.6	63.6	75.5	73.2	5.0
1 nM	137	138	152	77.8	78.4	86.3	80.8	2.7
10 nM	190	141	176	107.9	80.1	100	96.0	8.2
100 nM	196	170	151	111.3	96.5	85.7	97.9	7.4
1 µM	208	182	170	118.1	103.4	96.5	106.0	6.3
10 µM	197	180	188	111.9	102.2	106.8	107.0	2.7
100 µM	470	460	455	133.5	130.6	129.2	131.1	1.2
1 mM	0	0	0	0	0	0	0	0

3 Gy								
	Count	Count	Count	Survival	Survival	Survival	Mean Survival	SEM
Control	397	380	400	101.1	96.8	101.9	100	1.5
Media	378	374	420	96.3	95.3	107.0	99.5	3.7
Abs control	498	513	505	88.8	91.5	90.1	90.1	0.72
0.5 Gy	336	341	334	85.6	86.9	85.1	85.8	0.5
0 M	394	363	421	100.4	92.5	107.3	100.0	4.2
0.1 nM	383	343	348	97.6	87.4	88.7	91.2	3.2
1 nM	358	434	417	91.2	110.6	106.2	102.7	5.8
10 nM	397	348	362	101.1	88.7	92.2	94.0	3.7
100 nM	458	398	428	116.7	101.4	109.0	109.0	4.4
1 µM	199	212	195	74.8	79.6	73.3	75.9	1.9
10 µM	431	422	457	109.8	107.5	116.4	111.2	2.6
100 µM	409	394	451	104.2	100.4	114.9	106.5	4.3
1 mM	0	0	0	0	0	0	0	0

5 Gy								
	Count	Count	Count	Survival	Survival	Survival	Mean Survival	SEM
Control	352	370	336	99.8	104.9	95.2	100	2.7
Media	209	307	346	59.2	87.0	98.1	81.4	11.5
Abs control	352	369	342	99.8	104.6	96.9	100.4	2.2
0.5 Gy	174	173	200	49.3	49.0	56.7	51.7	2.5
0 M	411	400	380	116.5	113.4	107.7	112.5	2.5
0.1 nM	332	342	364	94.1	96.9	103.2	98.1	2.6
1 nM	398	394	405	112.8	111.7	114.8	113.1	0.9
10 nM	355	343	330	100.6	97.2	93.5	97.1	2.0
100 nM	343	328	369	97.2	93.0	104.6	98.2	3.3
1 µM	421	440	420	119.3	124.7	119.0	121.0	1.8
10 µM	367	380	383	104.0	107.7	108.6	106.8	1.3
100 µM	411	354	358	116.5	100.3	101.5	106.1	5.2
1 mM	0	0	0	0	0	0	0	0

Viability Assay

Background: 0.1907425

0 Gy								
	Abs	Abs	Abs	Abs-BG	Abs-BG	Abs-BG	Mean of corrected absorption	SEM
Control	1.0	0.7	0.9	0.8	0.5	0.8	0.7	0.1
Media	1.2	1.0	1.2	1.0	0.8	1.0	0.9	0.1
Abs Control	1.199	1.2	1.0	1.0	1.0	0.8	0.9	0.1
1 nM	1.3	1.4	1.1	1.1	1.2	1.0	1.1	0.1
10 nM	1.2	1.5	1.3	1.0	1.3	1.1	1.1	0.1
100 nM	1.3	1.0	1.1	1.1	0.89	0.9	1.0	0.1
1 µM	1.1	1.2	1.2	0.9	1.0	1.0	1.0	0.1
10 µM	1.2	1.4	1.2	1.0	1.2	1.1	1.1	0.1
100 µM	1.0	1.2	1.0	0.8	1.05	0.8	0.9	0.1

0.5 Gy								
	Abs	Abs	Abs	Abs-BG	Abs-BG	Abs-BG	Mean of corrected absorption	SEM
Control	1.0	0.7	0.9	0.8	0.5	0.8	0.7	0.1
Media	1.2	0.9	1.1	1.0	0.7	0.9	0.9	0.1
Abs Control	1.1	1.2	1.0	1.0	1.0	0.8	0.9	0.0
0.5 Gy Dose	1.3	1.6	1.3	1.1	1.4	1.1	1.2	0.1
1 nM	1.1	1.2	1.2	1.0	1.0	1.0	1.0	0.0
10 nM	1.4	1.1	1.0	1.2	1.0	0.8	1.0	0.1
100 nM	1.2	1.1	1.0	1.1	0.9	0.8	0.9	0.1
1 µM	1.5	1.3	1.1	1.3	1.1	1.0	1.1	0.1
10 µM	1.3	1.1	1.0	1.1	0.9	0.8	0.9	0.1
100 µM	1.5	2.0	1.2	1.3	1.8	1.0	1.4	0.2
5 Gy								
	Abs	Abs	Abs	Abs-BG	Abs-BG	Abs-BG	Mean of corrected absorption	SEM
Control	1.0	0.7	0.9	0.8	0.5	0.8	0.7	0.1
Media	1.1	1.2	1.0	0.9	1.0	0.9	0.9	0.0
Abs Control	1.1	1.2	1.0	1.0	1.01	0.85	0.9	0.0
5 Gy Dose	1.6	1.2	1.2	1.4	1.05	1.0	1.1	0.1
1 nM	1.0	1.1	1.0	0.9	1.0	0.8	0.9	0.0
10 nM	1.0	1.0	0.9	0.8	0.8	0.8	0.8	0.0
100 nM	1.0	1.1	1.2	0.8	0.9	1.1	0.9	0.1
1 µM	1.3	1.5	1.2	1.1	1.3	1.1	1.2	0.1
10 µM	1.3	1.2	1.2	1.1	1.0	1.0	1.0	0.1
100 µM	1.2	1.1	1.2	1.0	0.9	1.0	1.0	0.094

Adaptive Response

First Trial- 3 hrs								
	Count	Count	Count	Survival	Survival	Survival	Mean Survival	SEM
Control	161	165	194	92.8	95.1	111.9	100	5.9
0.1 Gy+5 Gy	23	13	14	26.5	15	16.1	19.2	3.6
5 Gy	13	18	11	15	20.7	12.6	16.1	2.4
1 nM+ 5 Gy	6	10	13	6.9	11.5	15	11.1	2.3
10 nM+ 5 Gy	16	9	9	18.4	10.3	10.3	13.0	2.6
100 nM+ 5 Gy	7	13	15	8.0	15	17.3	13.4	2.7
1000 nM+ 5 Gy	1	13	10	1.1	15	11.5	9.2	4.1
Second Trial- 3 hrs								
	Count	Count	Count	Survival	Survival	Survival	Mean Survival	SEM
Control	125	111	143	98.9	87.8	113.1	100	7.3
0.1 Gy+5 Gy	9	13	12	14.2	20.5	18.9	17.9	1.9
5 Gy	20	25	11	31.6	39.5	17.4	29.5	6.4
1 nM+ 5 Gy	13	14	1	20.5	22.1	1.5	14.7	6.6
10 nM+ 5 Gy	13	0	8	20.5	0	12.6	11.0	5.9
100 nM+ 5 Gy	7	8	3	11.0	12.6	4.7	9.49	2.4
1000 nM+ 5 Gy	4	3	12	6.3	4.7	18.9	10.0	4.5

First Trial- 12 hrs								
	Count	Count	Count	Survival	Survival	Survival	Mean Survival	SEM
Control	230	210	219	104.7	95.5	99.6	100	2.6
0.1 Gy+5 Gy	71	58	64	32.3	26.4	29.1	29.2	1.7
5 Gy	99	30	110	45.0	13.6	50.0	36.2	11.3
1 nM+ 5 Gy	41	51	55	18.6	23.2	25.0	22.3	1.8
10 nM+ 5 Gy	65	41	56	29.5	18.6	25.4	24.5	3.1
100 nM+ 5 Gy	51	82	64	23.2	37.3	29.1	29.8	4.0
1000 nM+ 5 Gy	79	51	41	35.9	23.2	18.6	25.9	5.1
2nd Trial- 12 hrs								
	Count	Count	Count	Survival	Survival	Survival	Mean Survival	SEM
Control	274	292	430	82.5	87.9	129.5	100	14.8
0.1 Gy+5 Gy	7	14	55	2.1	4.2	16.5	7.6	4.5
5 Gy	18	38	58	5.4	11.4	17.4	11.4	3.4
1 nM+ 5 Gy	10	8	4	3.0	2.4	1.2	2.2	0.5
10 nM+ 5 Gy	14	10	22	4.2	3.0	6.6	4.6	1.0
100 nM+ 5 Gy	19	3	5	5.7	0.9	1.5	2.7	1.5
1000 nM+ 5 Gy	12	15	26	3.6	4.51	7.8	5.3	1.2

Nicotine and Radiation-induced Bystander Effect in RT-112

	Counts	Counts	Counts	Survival	Survival	Survival	Mean Survival	SEM
Control	79	81	79	99.1	101.6	99.1	100	0.8
Media Control	82	83	84	102.9	104.1	105.4	104.1	0.7
Sham Irradiation	68	78	81	85.3	97.9	101.6	94.9	4.9
Bystander	51	61	59	64.0	76.5	74.0	71.5	3.8
0.5 Gy dose	108	106	115	135.5	133.0	144.3	137.6	3.4