Abstract

Reactive oxygen species (ROS) are chemically reactive molecules that are naturally produced within the biological system. The accumulation of ROS is controlled by antioxidant defense. Low levels of ROS act as important signaling messengers mediating cell apoptosis, inflammation and immune responses. However, this redox balance can be disturbed by pathological assaults, in which excessive ROS are generated, leading to oxidative damage and dysregulated cellular activities.

The positive aspects of ROS as signaling molecules have been exploited in ischemic and hypoxic preconditioning; while the negative roles of ROS in mediating cell apoptosis and inducing oxidative damage have been extensively studied in cancer treatment. Particularly, as shown in my first project, ROS play a role in radiation-induced bystander effects, the phenomenon where irradiated cells can emit signals to affect neighboring cells. Due to such effects, a gradient dosage design demonstrates the potential to achieve a similar tumor killing efficiency as the uniform-dose profile but with less radiotoxicity on neighboring normal tissues. To examine the effectiveness of gradient irradiation in tumor curbing, we monitored the cell viability, intra- and extra-cellular ROS production patterns within 48h following gradient (8-2 Gy) and uniform (5 Gy) irradiation in breast cancer cells (MCF-7). Similar levels of ROS and lower cell viability were observed in the gradient irradiation group as compared to uniform irradiation group.
at 48h after irradiation. Our results suggest superior therapeutic effects of gradient irradiation, which promises great potential to be used in current radiotherapy for the benefits of cancer patients.

My second project is to explore the molecular mechanism of hypoxic preconditioning (HPC), as HPC can exert strong protection on skeletal muscles that are exposed to hypoxia and the following reoxygenation; yet the underlying mechanisms remain elusive. HPC treatment is likely to induce changes in muscle redox status, which may improve muscle resistance to hypoxia through ROS-initiated pathways and Ca\(^{2+}\) handling. NADH is a critical mitochondrial redox indicator; potentially it is linked with ROS formation and Ca\(^{2+}\) release. Therefore, we used a photometer system to characterize the NADH fluctuations in HPC-treated mouse diaphragms. The results may provide useful insights into the protective mechanisms of HPC.
Dedication

This document is dedicated to my family.
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Vita

July, 2013................................. B.S. Foreign Transportation,
Dalian Maritime University, Dalian, China

January, 2016 to Present....................... Graduate Research Assistant,
School of Health and Rehabilitation Sciences, The Ohio State University

Publications

formation and bystander effects in gradient irradiation on human breast cancer cells.

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Chapter 1 Introduction

1.1 Physiological Sources of ROS

ROS are chemically reactive molecules that include oxygen (O$_2$)-containing free radicals and non-radical O$_2$ derivatives [1]. Common examples of ROS are superoxide (O$_2$\(^{-}\)), hydroxyl radical (\(\cdot\)OH), peroxynitrite (ONOO\(^{-}\)), and hydrogen peroxide (H$_2$O$_2$), all of which are naturally produced within biological systems [2]. ROS are generally impermeable to membranes due to their polarity; thus, intracellular and extracellular ROS are presumably generated along separate pathways [3]. Under normal physiological conditions, ROS can be produced as the byproducts of mitochondrial respiration. O$_2$ in mitochondria gains one electron through electron leakage within the respiratory chain and generate O$_2$\(^{-}\) [4]. Therefore, the interruption of mitochondria usually precedes an ROS increment [5]. In addition, NADPH oxidases (Nox) act as a major ROS generator (typically O$_2$\(^{-}\)) [6]. Nox are located within the plasma membranes and use NAD(P)H as their major electron donors to transfer cytoplasmic electrons to extracellular O$_2$ and produce O$_2$\(^{-}\) [7, 8]. ROS formation has also been shown correlated with arachidonic acids (AA) metabolism. AA initiate lipoxygenase and cytochrome P450-dependent monooxygenase pathways, leading to ROS formation [6].
1.2 Physiological Functions of ROS

ROS mainly function as signaling molecules mediating activities including cell apoptosis, vascular contraction/relaxation, and inflammation and immune response [6, 8]. For instance, Nox-induced ROS play an important role in mediating endothelial activities and controlling vascular contraction-relaxation. The activities of Nox are regulated by several growth factors, cytokines and physical factors such as shear stress [8]. Low levels of ROS are good for cell function; however increased ROS may trigger programmed cell death [6, 9]. ROS act as key mediators involved in cell death receptors signaling (e.g., Fas and tumor necrosis factor (TNF) receptors) and also initiate apoptosis by causing mitochondrial membrane permeabilization and release of cytochrome c [10].

Furthermore, H$_2$O$_2$ (a major ROS) can stimulate cellular apoptosis in a c-Abl/Arg-dependent pathway. When cells are exposed to H$_2$O$_2$ at a low concentration, c-Abl and Arg, crucial types of tyrosine kinases enhance the activity of catalase (a crucial antioxidant) via phosphorylation process and thus protect cells against oxidative damage [11-13]. However, as the H$_2$O$_2$ concentration increases, both c-Abl and Arg start to separate from catalase, resulting in the deactivation of catalase and apoptosis initiation [12-14]. In this cycle, H$_2$O$_2$ is engaged in the mediation of cellular functions through the control of catalase activity. The simplified c-Abl/Arg mediation mechanism is described in Fig. 1 [12, 13, 15].
The roles of ROS are also well established in inflammation, as they are involved in the activation of Nod-like receptors (NLRs) inflammasome [6]. An elevation of ROS is implicated in the progression of chronic inflammatory diseases such as asthma and chronic obstructive pulmonary disease (COPD) [16]. Some inflammatory cells, such as mast cells, macrophages, and neutrophils have been shown to boost ROS production when activated by external stimuli (e.g., pollution particles) [17, 18]. Excessive ROS accumulation precipitates the inflammatory condition, leading to oxidative stress (OS) and airway hyper-responsiveness, as manifested in asthma patients [16]. Recent studies have revealed the potential roles of mitochondrial ROS in the generation of several pro-inflammatory cytokines driven by lipopolysaccharide (LPS) [19]. In addition, ROS make up part of the defense system against external microbes. For instance, eosinophil
peroxidase (EPO, a type of antioxidant) use halides as its reductive substrate for the decomposition of H₂O₂ to generate hypohalous acids [20]. These strong cytotoxic products accounts for the significant antibiotic effect [21].

1.3 Antioxidant Defense and Oxidative Stress (OS)

Under normal physiological conditions, ROS formation is kept at a low level by antioxidant defense system. Various antioxidants work in a synergistic way to control ROS concentrations and ensure normal cellular functions. O₂⁻ is naturally produced within the biological system and formed in excess in pathologic circumstances [6]. O₂⁻ can react with nitric oxide (’NO) to produce peroxynitrite (ONOO’), which exerts highly toxic effects on cellular components [22]. Superoxide dismutase (SOD) is major O₂⁻ scavenger. In the presence of H⁺, O₂⁻ is rapidly converted by SOD to H₂O₂, which is less reactive, via reaction (1) as shown below at a rate of 2 × 10⁹ M⁻¹s⁻¹:

\[
2O_2^- (+ 2H^+) \xrightarrow{\text{SOD}} O_2 + H_2O_2
\]

In human and the other mammals, three types of SOD (SOD1, SOD2 and SOD3) have been identified according to their locations [23-25]. SOD1, a Cu–Zn-cofactored dimer, is commonly found in the cytoplasm [25-27]; SOD2, a tetramer cofactored with Mn, is found in mitochondria [28, 29]; and SOD3, a Cu–Zn-cofactored tetramer, is located extracellularly [24]. Deficiency in any type of SOD would result in decreased resistance to oxidative stress (OS), leading to tissue damage [6, 30-32].
Although H$_2$O$_2$ is a relatively stable product as compared to O$_2$·⁻, it can produce ·OH via the Fenton reaction in the aqueous solution at a pH of 3-8 (equation 2) [33, 34]. ·OH is a highly reactive molecule that indiscriminately oxidizes most organic macromolecules it contacts at a diffusion-limit rate (~ 10$^9$-10$^{10}$ M$^{-1}$ s$^{-1}$), causing severe biological damage [35].

\[ \text{L-Fe (II) + H}_2\text{O}_2 \rightarrow \text{L-Fe (III)} + \text{OH}^- + \cdot \text{OH} \]  \hspace{1cm} (2)

There are several crucial H$_2$O$_2$ scavengers in the body including catalase, glutathione peroxidase (GPx), and peroxiredoxin (Prx) [6]. Catalase is primarily found in peroxisomes and decomposes H$_2$O$_2$ into H$_2$O and O$_2$ (equation 3) [36].

\[ 2\text{H}_2\text{O}_2 \xrightarrow{\text{catalase}} 2\text{H}_2\text{O} + \text{O}_2 \]  \hspace{1cm} (3)

Due to the universal expression, catalase provides essential protection for virtually every organism [6, 37-39]. In a catalase knock-out mouse model established by Ho et al. in 2004, catalase-absent mice developed no prominent defect under normal conditions; however, when specific oxidative injuries were imposed on various tissues such as the liver, lung and brain, these mice showed different levels of susceptibility to OS. Therefore, protection exerted by catalase is considered as tissue dependent and influenced by the presence of the other antioxidant such as GPx [37]. GPx is a family of potent antioxidant enzymes that are mainly responsible for scavenging excessive H$_2$O$_2$ (equation 4) as well as reducing lipid hydroperoxide using glutathione (GSH) as its substrates [40-43].
\[
\text{H}_2\text{O}_2 + 2\text{GSH} \xrightarrow{\text{GPx}} 2\text{H}_2\text{O} + \text{GS} - \text{SG}
\]

Of the eight GPx variants (i.e. GPx1–8) that have been identified, GPx1 is among the most abundant antioxidant enzymes in human tissues [44]. Generally, it exerts a strong protective effect in aqueous areas of the cell and is considered to play a complementary role in peroxide reduction with vitamin E, a lipid-soluble peroxide scavenger [45]. On the other hand, GPx4 (or hydroperoxide GPx), is highly efficient in inactivating phospholipid peroxidation, thus exerting protective effects on both mitochondrial and plasma membranes [6, 46-49].

Other biological antioxidant defense includes Prx, EPO, and thioredoxin (Trx) [6]. Prx is a potent peroxidase distinguished by its extensive distribution among various species and in nearly all tissues [13, 50, 51]. Essentially, it utilizes a thiol group to reduce peroxide [52]. Although Prx demonstrates a much lower catalytic efficiency as compared to GPx and catalase, its universal distribution is a piece of convincing evidence for its importance as an antioxidant [51, 52]. Trx plays a role in the mediation of other antioxidant actions [6, 53]. For instance, it is involved in the regeneration of lipoic acid, ubiquinone, selenium-containing substances and ascorbic acid (vitamin C) [54]. Such interplays between Trx and other antioxidants are marked in the regulation of redox homeostasis within the body. Furthermore, vitamins A, C, D, E, β-carotene, selenium and manganese also demonstrate the abilities to scavenge ROS [6, 55-57].

Under normal physiological conditions, antioxidants are important in protecting the body against ROS assault. However, external stimuli or diseases are likely to induce
excessive ROS formation, resulting in OS [6]. Prolonged OS is observed under a wide range of pathological conditions, such as inflammation, neurodegenerative disorders, and ischemia reperfusion (I/R) injuries. Associated oxidative damages are manifested as DNA mutations, protein dysfunction, and lipid peroxidation. Accordingly, antioxidant treatments have been referred as a potential way of lessening OS-induced damage; however, mixed results have been yielded regarding its effectiveness to alleviate disease symptoms. This is possibly due to a lack of understanding of the interplay of various biological antioxidants and a neglect of the beneficial roles of ROS [6]. The intriguing roles of ROS and different antioxidants in the context of specific pathological circumstances and therapies will be intensively discussed in Chapter 2.
Chapter 2 The Good and Bad of ROS

2.1 The Bad of ROS

ROS such as $\text{O}_2^-$, 'OH, and ONOO' are highly reactive molecules that can cause oxidative damage in DNA, proteins and lipids [6]. Excessive ROS accumulation has been implicated in a range of pathological conditions as well as aging. Increased OS is thought to be a determinant for aging process. More ROS are often observed in aged populations, possibly due to the decreased antioxidant activities and the compromised organelle function, such as mitochondria, a major source of ROS [58]. Moreover, high levels of ROS have been linked to neurologic and psychiatric diseases such as Parkinson’s disease and Amyotrophic lateral sclerosis (ALS) [59-61]. OS is indicated as a critical pathological factor of Parkinson’s disease by targeting dopaminergic neurons [61]. Other studies have found that the SOD1 knocked-out mice developed motor neuron degeneration symptoms as observed in ALS patients while the Nox1- mutation can increase the lifespan of SOD deficiency mice, indicating the possible relevance of Nox to ALS [61]. Furthermore, high level ROS play an important role in inflammatory diseases (e.g., COPD and asthma), I/R injuries and cancer [6]. Persistent OS causes DNA mutations, thereby increasing cancer occurrence [62]. Thus, the key roles of ROS in asthma and mitochondrial DNA mutations will be thoroughly discussed in the following sections.
2.1.1 Redox Mechanisms in Asthma

Asthma is a long-term airway inflammatory disorder, defined by symptoms including wheezing, coughing, and shortness of breath. Typical pathophysiological changes of asthma are associated with reversible airflow limitation and airway hyper-reactivity. However, long-standing asthma results in permanent airway remodeling [63, 64]. Reversible symptoms vary with time and may even become undetectable without medication. The underlying pathological presentations such as inflammation and hyper-responsiveness usually persist, even in the absence of symptoms or with normal lung functions [63].

Based on the report by the Asthma and Allergy Foundation of America (AAFA), over 300 million people are suffering from this disorder worldwide [16]. Several types of asthma have been defined by their phenotypes including allergic asthma, non-allergic asthma, and obesity-related asthma [63]. Allergic asthma, most commonly found in the childhood, is characterized by eosinophilic airway inflammation, usually correlated with a familial history of allergic diseases. On the other hand, non-allergic asthma is mostly seen in adults. The inflammatory cells present in these asthmatics include neutrophils and eosinophils. Certain asthma patients with obesity demonstrate significant respiratory symptoms while maintaining low levels of eosinophilic airway inflammation [63].

During acute severe asthma, interleukin (IL)-8 mediates the recruitment of neutrophils to the airway, leading to substantial inflammation [65]. Other inflammatory cells including mast cells, eosinophils, and macrophages are also stimulated upon
exposure to allergens [16]. The accumulation of these inflammatory cells initiates excessive production of $\text{O}_2^{•–}$ and $\text{H}_2\text{O}_2$, which may be implicated in the signaling cascade of asthma [16, 66, 67]. For instance, ROS can activate NF-κB in epithelial cells, promoting the expression of pro-inflammatory cytokines and adhesion molecules [16, 66]. Increased ROS formation plays a critical role in mediating the activity of TNF-α and IL-1 [16]. These mediators can further modulate other pro-inflammatory cytokines such as vascular cell adhesion molecules and intercellular adhesion molecule-1, which are implicated in the pathogenesis of asthma [16, 68]. Moreover, Nox is a critical source of ROS in asthma. The Nox-derived ROS can trigger the production of immunoglobulin E (IgE), which acts as an important mediator to initiate the hypersensitive responses in the airway [16].

Healthy lungs normally prefer a more reducing environment, due to a robust antioxidant system [64]. However, the redox balance can be destabilized in asthma patients because of the excessive ROS accumulation and compromised antioxidant activities [16, 64]. The ratio of GSH/glutathione disulfide (GSSG), a critical redox indicator, is markedly lowered in epithelial lining fluid [64]. Substantial oxidative damages such as DNA mutation, protein modification, and lipid peroxidation are observed in asthma lungs [16]. Specifically, a highly oxidative environment can lead to the inefficacy of antioxidant enzymes thus exacerbating OS conditions [64]. Studies have revealed markedly decreased SOD and catalase activities in asthma subjects [64]. Altered intracellular redox conditions can stimulate mitogen-activated protein kinase (MAPK)
signaling including c-jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), further facilitating the inflammatory process [64].

Reactive nitrogen species (RNS) also play a role in the pathogenesis of asthma [69]. Increased \( \cdot \text{NO} \) production is associated with enhanced nitric oxide synthase (NOS) activity in the lung [64, 69]. \( \cdot \text{NO} \) can react with \( \text{O}_2^{\cdot} \) to form \( \text{ONOO}^- \), a highly toxic molecule that results in tyrosine nitration and subsequent respiratory tract damage [64, 69]. Accordingly, therapies regulating the redox state in the airway may be beneficial in asthma control.

2.1.2 ROS and Mitochondrial DNA Damage

Mitochondria are well known for their certain hereditary independence by possessing unique DNA (mtDNA) and protein synthesis system [70, 71]. Most of the proteins located in mitochondria are indeed encoded by nuclear DNA (nDNA) [70]. The proteins translated from nDNA are transported into mitochondria and participate in the assembly of mitochondrial complex, along with mtDNA-encoded proteins [72]. Human mtDNA is mainly maternally inherited despite the fact that the sperm contributes around 100 mitochondria during fertilization. Increased levels of mutated mtDNA and the subsequent mitochondrial dysfunction are frequently observed in the development of multiple mtDNA-related diseases [73]. High mtDNA mutation rate contributes to the genetic polymorphism in the population, and it is likely linked to both human evolution and migration [73, 74]. Mutations in the mtDNA may lead to significant biological defects, compromising essential respiratory chain and impairing ATP bioenergetics [75].
Both endogenous factors such as ROS and environmental exposures can potentially induce mtDNA damage, resulting in mitochondrial diseases when the damage is beyond repair.

The genetic aspect of mitochondria is quite complicated partially due to its continuous degradation and replenishment [76]. The respiratory chain in mitochondria generates large amounts of ROS, which exert frequent oxidative attack on DNA [77-79]. The nonchromatinized structure and the absence of histones contribute to the extensive susceptibility of mtDNA to any potential genetic mutation [77]. A practical indicator of oxidative DNA damage, 8'-hydroxy-2'-deoxyguanosine (Oh8dG), has been shown to exist at a higher level (one per 8,000 bases) in mtDNA as compared to nDNA (one per 130,000 bases) [80]. Moreover, the insufficient repair mechanism of mtDNA increases the possibility of irreversible mutation in mtDNA [81]. The exact mechanism of mtDNA mutation is not fully elucidated, yet the frequent occurrences of mtDNA deletion have been suggested correlated with processes such as slipped mispairing during the replication [82].

Oxidative-induced DNA alteration accounts for the majority of DNA damage, which is more profound in mitochondria. The endogenous ROS can attack mtDNA, resulting in the generation of DNA adducts [83]. Yakes and Van Houten utilized PCR techniques to quantitatively monitor hydrogen peroxide (H2O2)-induced mutations in both mtDNA and nDNA fragments. They observed three-fold mtDNA damage as compared to nuclear genome after H2O2 treatment. Following 60-min incubation in fresh conditioned medium, the oxidative damage in nDNA was significantly alleviated due to
recovery. However, little repair was observed in mtDNA [84]. Yakes and Van Houten proposed that this persistent mtDNA damage may be associated with the formation of secondary ROS, including the byproducts of lipid peroxidation [84]. Research has further discussed the role of ROS-related factors such as tumor TNF-α in the mechanism of mtDNA impairment [85]. It has been suggested that TNF-α can induce mtDNA damage via the production of excessive ROS at the mitochondrial inner membrane, which subsequently contributes to the declined complex III activity. Such damage in mtDNA can be effectively prevented by the administration of antioxidants [85]. Due to the remarkable correlation between mtDNA mutation and oxidative stress, mtDNA damage has been implicated as a potential biomarker for ROS-associated diseases [84]. Accordingly, OS that is elicited by defective mitochondria is likely to participate in the natural aging process. The accumulation of mutated mtDNA and ROS throughout the lifespan can be regarded as contributing to the mechanisms for aging and age-related neurodegenerative disorders such as ALS and Parkinson’s disease [86, 87].

The high proportion of mutations in mtDNA can be detected in aging, tumor cells and mitochondria-related diseases [76]. The mitochondrial dysfunction due to aging may cause excessive ROS formation, accompanied by a reduced antioxidant activity, fostering the oxidative-induced mtDNA mutation [82]. Interestingly, Kujoth et al. have found that mtDNA mutation was correlated with the overexpression of apoptotic markers in aged mice [88]. For instance, the persistent single-strand breaks in mtDNA can promote ROS formation, triggering the initial apoptosis process [77]. In tumor cells, seemingly neutral mutations may contribute to the neoplasm via clonal expansion while mitochondrial-
related diseases are usually characterized by high proportion of mutated mtDNA [76]. Additionally, environmental factors such as ultraviolet irradiation, smoking and alcohol, can potentially induce mtDNA mutations [82, 89]. The mtDNA adducts generated upon environmental exposures can interfere mitochondrial transcription and replication. The gene expression of mitochondria is also hindered due to environmentally-induced ROS and the chemical modifications of mtDNA [89]. MtDNA degradation is a crucial step of maintenance when damage is well beyond the repair process [90]. Persistent mtDNA damage can also disrupt the respiratory chain, resulting in excess ROS production and ultimately cell apoptosis [91]. For instance, Tann et al. found that the overproduction of single-strand breaks in mtDNA elevate ROS levels, triggering the apoptotic pathway [77].

2.2 The Good of ROS

Although high levels of ROS are associated with some disease progression, therapies that utilize antioxidants to scavenge ROS may diminish their beneficial effects as key signaling molecules [6, 92]. In fact, triggering of ROS production has been implicated in several crucial therapeutic approaches including ischemic preconditioning (IPC). A small amount of ROS induced by IPC activates a series of protective pathways in cardiomyocytes during the subsequent I/R stages [92]. Moreover, since overproduction of ROS can cause oxidative damage and initiate apoptotic pathways, the role of ROS to induce cell death has been extensively explored in cancer treatment [6, 93]. The following sections will focus on the beneficial effects of ROS in the context of IPC, ischemic postconditioning, and cancer treatment.
2.2.1 Protective Roles of ROS in Myocardial I/R Injuries

Myocardial I/R injuries occur when blood is reintroduced to the ischemic region of heart, which is commonly found in coronary heart diseases and peri-operative period [94]. Evidence has shown that a burst of ROS occurs on the onset of ischemia as well as during the following reperfusion stage. Excessive ROS production has been indicated as a primary contributor of I/R injury [95]. Charles et al. first described the protective effect of preconditioning on myocardium as early as 1986 as they observed a slower ATP depletion rate and smaller infarct size in the heart treated with brief episodes of I/R cycles before occlusion [96, 97]. Later research recognized several types of preconditioning protocols including IPC, exercise preconditioning, and pharmacological preconditioning [98-100]. Preconditioning can provide a beneficial “warm-up” that basically primes the tissue to subsequent injuries caused by prolonged stresses such as ischemia and hypoxia [98, 101]. Small amounts of ROS generated during short periods/cycles of I/R are indeed associated with the protective effect exerted by preconditioning [98]. In particular, ROS originated from the mitochondria play a pivotal role in mediating cardioprotection via mechanisms involving the activation of survival programs [98, 102]. Moreover, lower OS has been observed in I/R preconditioned cardiac muscle during prolonged I/R, which is attributed to the reduced ROS generation in mitochondria [98]. One of the well-established IPC mechanisms involves the opening of mitochondrial ATP-sensitive K+ (mitoK$_{ATP}$) channel. MitoK$_{ATP}$ channel is activated upon the exposure to preconditioning stimuli while the subsequent influx of K$^+$ leads to depolarization and matrix alkalization, which consequently induces a moderate increase in ROS and the activation of
downstream survival signaling events [102, 103]. Notably, these preconditioning-induced ROS may mediate protein kinase C (PKC) activity and, via multiple steps, inhibit the opening of mitochondrial permeability transition pore (mPTP) [98, 102]. As discussed earlier, mPTP is a major regulator of necrosis and apoptosis [104], such inhibition of mPTP opening is therefore essential to the cardioprotection [98]. In addition, the opening of the mitoK$_{ATP}$ channel can generate mild matrix swelling which can improve ATP synthesis and fatty acid oxidation, leading to cardioprotective effects [98]. The application of mitoK$_{ATP}$ openers mimic IPC whereas K$_{ATP}$ blockers, such as 5-hydroxydecanoate, attenuate cardioprotection further suggesting the importance of mitoK$_{ATP}$ in IPC protection [103]. Besides the exclusive role of mitochondrial ROS in signaling IPC [102], the initial burst of ROS is correlated with IPC efficacy, and it serves as essential preconditioning stimulus to the activation of mitoK$_{ATP}$ as well as sarcolemmal K$_{ATP}$ (sarcK$_{ATP}$) [92, 98, 105].

Ischemic postconditioning, mentioned earlier by Zhao et al. in 2003, demonstrates a cardioprotection that is tantamount to IPC in a non-pretreated heart after I/R [103]. It is later defined as “brief periods of ischemia alternating with short periods of reflow applied at the onset of reperfusion following sustained ischemia” [106, 107]. Since reperfusion injuries occur within several minutes of blood reflow, postconditioning must be introduced as soon as the reperfusion is initiated [107]. Basically, postconditioning and preconditioning follow similar protocol in which the myocardium is exposed to cycles of ischemia and reperfusion; however, the timing when the treatment is performed varies. In a rat I/R model established by Kin et al., three postconditioning cycles were performed at
the onset of reperfusion. Each cycle consisted of 10 s reperfusion followed by 10 s reocclusion. This postconditioning protocol largely decreased I/R-induced damage, but demonstrated less cardioprotection as compared to preconditioning, which consisted of five min-ischemia/10 min-reperfusion cycles before the initiation of occlusion [108]. Although the extent of postconditioning in attenuating reperfusion injury remains elusive [109], it is clear that the duration of reperfusion-ischemia cycles and the number of cycles greatly influence the degree of the protective effect [107]. For instance, in a 30 min occlusion model, rats that were treated with three cycles of 30 s reperfusion and ischemia had less infarct size. However, detrimental effects occurred when the duration of treatment in each cycle was down to 5 or 15 s [110]. The protective effect of postconditioning has demonstrated a similar mechanism to preconditioning, in which ROS are readily involved. ROS generation during early reperfusion was found to play an essential role in initiating the protective cascade, possibly via the activation of mitoK_{ATP}. The mitoK_{ATP} opening raises the level of H_{2}O_{2}, which ultimately leads to mPTP inhibition and thus prevents cell apoptosis [107]. Currently, the effect of postconditioning against I/R has yielded variable results and further research is necessary to evaluate its protection on the heart [92].

2.2.2 ROS-Targeted Treatment of I/R Injuries

Despite the beneficial role of ROS in preconditioning [95], excess ROS have been implicated in the pathogenesis of I/R injury and increasing studies have been focusing on developing potential therapies to prevent ROS accumulation [102]. In particular, site-targeted treatments such as inhibiting ROS generation by mitochondria or Nox may
improve the protective effect on the stressed myocardium [95, 111]. Furthermore, the combination of different antioxidants has been found effective in resisting I/R injuries [95]. Gao et al. have reported that glutathione provides better cardioprotection than ascorbic acid when treated at the beginning of reperfusion in a rat heart model. Moreover, the co-administration of both antioxidants enhances the protective effect as compared to individual treatment [112]. One study examined the effect of VitaePro, a mixed antioxidant compound, and vitamin E in a 21-day oral treatment on rats before the induction of I/R. The results showed that both VitaePro as well as Vitamin E exert cardioprotective effects during I/R, while VitaePro demonstrated a much stronger effect. The work suggests potential prospects of antioxidant drugs in resisting I/R injury [92, 113].

Apart from antioxidant treatments that scavenge excess ROS, targeting inhibition of ROS production at their own sources may be a more favorable approach. With the sole function of generating ROS in both physiology and disease states [114], Nox have been considered as a therapeutic target in ROS-related injuries. Nox attributes to a portion of ROS production during reperfusion [115]. In response to I/R injury, both Nox2 and Nox4 isoforms are upregulated in the heart [116]. Although it is reasonable to inhibit Nox activities in order to lessen the ROS activation during I/R injury, a complete inhibition of Nox is not ideal since Nox is responsible for the physiological production of ROS. Indeed, a minimal amount of ROS is essential to prevent I/R injury via metabolic adaptations involving hypoxia-inducible factor-1α (HIF-1α) and peroxisome proliferator-activated receptor-α (PPARα)-dependent mechanisms [116]. Therefore, selective
blockage of Nox is highly desirable, yet the development of such isoform-specific Nox inhibitor prompts more difficulties [114, 116]. Unfortunately, chemical inhibitors such as apocynin (a common Nox2 inhibitor) and diphenyleneiodonium have failed to achieve sufficient specificity [116]. In addition to Nox, accumulated evidence also suggests the involvement of XO in myocardial I/R oxidative injuries [117]. Studies evaluating allopurinol, a potent XO inhibitor, have generated positive results on reducing ROS generation and inhibiting cardiomyocyte apoptosis in myocardial infarction models [92, 118, 119].

Other pharmacologic agents can be used to stimulate conditioning pathways, in order to be as effective as preconditioning treatments. For instance, adenosine reduces the myocardial infarct size by activating cardiomyocyte receptors and subsequent PKC pathways that are involved in preconditioning-induced cardioprotection [120]. Cyclosporin administration before or at the onset of reperfusion also significantly ameliorates I/R injury by inhibiting mPTP opening and reserving mitochondrial function [115, 120]. Furthermore, drugs targeting the activation of K_{ATP} channels, such as nicorandil and pioglitazone, have been shown to demonstrate prominent cardioprotection against I/R-induced injuries [121]. Therefore, activating specific conditioning cascade sites by corresponding drugs highlighted potential treatments to achieve similar protective effect as preconditioning [92].
2.2.3 ROS in Cancer Treatment

Chronic hypoxia is a key characteristic manifested in most tumors, which can lead to mitochondrial ROS accumulation. The high levels of ROS in malignant cells are involved in the signaling of several growth factor receptors. For instance, increased ROS may contribute to hypoxic adaption, cell proliferation as well as apoptosis suppression in tumor cells [122]. Therefore, diminished ROS may affect tumor progression via the inhibition of redox signaling benefits. However, the attempt to restrain tumor progression by blocking ROS formation has not yielded successful clinical outcomes, possibly due to the complex mechanisms underlying cancer growth. On the other hand, it is well known that ROS formation that exceeds a known threshold can induce cell apoptosis [10]. Increasing studies have been focused on the oxidative toxicity of ROS for cancer treatments. Associated pharmaceutic agents include antioxidant inhibitors such as disulfiram (a Cu, Zn-SOD inhibitor) and buthionine sulfoximine, which can deplete the pool of GSH [122]. Furthermore, since cellular ROS accumulation can increase the radiosensitivity in tumors, a combination of antioxidant interruption and traditional radiotherapy (RT) may represent an attractive aspect for future research. For instance, Yang et al. proposed the concept of using antioxidant inhibitors to interrupt the redox balance in cancer cells [93]. Cancer cells are usually equipped with a stronger antioxidant defense in response to higher ROS levels after malignant transformation. For instance, PrxI (a H$_2$O$_2$ scavenger) has been found overexpressed in various cancer cells. The study thus utilized a potent PrxI inhibitor, AMRI-59, to treat A549 human lung carcinoma cells.
Their results reveal a significant anti-tumor effect of AMRI-59 with no acute side effect on normal cells (Fig. 2) [93].

Photodynamic therapy (PDT) is another clinical treatment for cancer that utilizes the cytotoxicity of ROS to be a supplementation of conventional RT [123]. Non-toxic photosensitizer molecules are delivered to tumor site. When excited by X-ray or UV light, these non-toxic molecules can generate a large amount of toxic ROS such as singlet oxygen (¹O₂) and other free radicals, [123-125]. Indeed, PDT has been tested in a wide range of cancers, yielding some positive outcomes. However, the success is largely limited by the specificity and potency of the treatment. The localization of tumor site by photosensitizer is a key issue to determine PDT efficiency. How to increase the selectivity of photosensitizers is the main focus of PDT studies in the past years. Specific carriers such as conjugated antibodies have been developed in order for photosensitizers to be selectively delivered to the treatment site [125].

Furthermore, ROS are key mediators of cell death induced by ionizing radiation (IR). IR instantaneously induces water radiolysis to generate ROS such as ‘OH, hydroperoxyl radical (HO₂), and H₂O₂. These short-lived molecules result in DNA attack and curbing tumor progression [126-128]. Recent studies have confirmed the secondary production of ROS from mitochondria in irradiated cells, which are implicated in cellular responses especially to low-dose irradiations. For instance, depolarization of mitochondrial membrane potential and the significant arrest of cells in G2/M phase were observed in coincidence with mitochondrial ROS formation following IR [126, 128]. Yamamori et al. have found that the cells accumulated in the G2/M are characterized by a
higher cellular OS, suggesting the possible contribution of cells in G2/M phase to IR-induced mitochondrial ROS release [128]. On the other hand, Leach et al. have proposed a calcium (Ca\(^{2+}\))– and mPTP– dependent mechanism in mediating ROS generation following IR. This was based on the fact that the application of mPTP inhibitor (cyclosporine A) and Ca\(^{2+}\) chelator effectively inhibited IR-induced ROS overproduction and mitochondrial membrane potential dissipations [126]. Due to these crucial roles of ROS in mediating cellular activities in response to IR, a more profound understanding of IR-induced biological ROS formation may help optimize the current RT.
Figure 2. A schematic illustrating the potential mechanisms of cancer killing effects by using PrxI inhibitor (AMRI-59). Prx, Peroxiredoxin [94].
Chapter 3 ROS in Bystander Effects and Radiotherapy: Real-World Experimental Approaches

3.1 Introduction

Previous studies have shown that irradiated cells can emit signals to affect neighboring cells, termed “bystander effects”. Cells that receive bystander signals suffer from adverse effects similar to those experienced by irradiated cells (i.e., genomic instability and apoptosis) [129, 130]. Bystander effects are evidenced by the fact that the medium collected from irradiated cells or from its progeny can induce apoptotic cascades in un-irradiated cells manifested as transient intracellular Ca^{2+} increase, sustained OS and depolarization of mitochondrial membrane potential [131]. Moreover, it has been found that serum harvested from patients who have received RT also led to cell death and genomic damages in un-irradiated cultured cell lines [129]. Multiple mechanisms, such as ROS- or inflammatory cytokines-medicated cellular pathways, have been proposed to explain such phenomenon [130]. However, it remains unknown how bystander signals are transferred from irradiated cells to low dose- or un-irradiated cells. Current RT is developed based on the uniform-dose models. The consideration of bystander effects may improve conventional RT by delivering non-uniform but lower dose to tumor targets, which can be designed to spare radiation toxicity to nearby healthy cells [130]. This introduces a
gradient dose design, which will be compared with uniform irradiation in terms of their ROS production pattern and the cell killing effects [130].

3.2 Methods

3.2.1 Cell culture and irradiation experiments

MCF-7 cell line was purchased from Cell Biolabs (San Diego, CA) and cultured on the BD Matrigel (Becton, Dickinson and Company (BD), Franklin Lakes, NJ, USA)-coated dish (3 cm diameter) two days before irradiation. Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Carlsbad, CA, USA) with 10% FBS (Life Technologies, Carlsbad, CA, USA) and 1% penicillin (Life Technologies, Carlsbad, CA, USA) were used as cell culture media. After cells uniformly adhered to the plate, irradiation was delivered. Cultured MCF-7 cell dishes were placed on the custom-made acrylic block to receive irradiation. These blocks were designed to contain three cell plate holders, as shown in Fig. 3A. Solid water slabs were placed underneath the plate holder to provide backscatter, as shown in Fig. 3B. The plate holder and solid water slabs were scanned together using a Siemens SOMATOM Sensation Open Syngo CT scanner (Siemens Medical Solutions, Mode #: 49445, Erlangen, Germany). Varian Eclipse treatment planning system (Varian Medical Systems, version 10.0.42, Palo Alto, California, USA) was used to design the irradiation profile, calculated at the red line location as shown in Fig. 3B [130].

Under GI, each plate was irradiated by three fields (Fig. 3C): Field 1, field size 1 cm x 1 cm, 500 MU, dose rate 600 cGy/min, energy 6MV; Field 2, field size 2 cm x 2
cm, 400 MU, dose rate 600 cGy/min, energy 6MV; Field 3, field size 5 cm x 5 cm, 200MU, dose rate 600 cGy/min, energy 6MV. Overlaps of these three fields (the purple line in Fig. 3C) generated a gradient dosage profile ranging from ~ 8 Gy at dish center decreasing to ~ 2 Gy at the edge of the dish (1.5 cm radius). Three typical irradiation bands were defined based on their distance from the center of the culture dish (Fig. 3D): i) Central 0.5 cm-radius circles were defined as 1 cm circles; ii) Circular bands between 0.5 cm and 1.0 cm were defined as the 2 cm bands; iii) Circular bands between 1.0 cm and 1.5 cm were defined as the 3 cm bands. The estimated volume-average dose for the three bands, using normalized integral dose in the corresponding band range, were 7.3 Gy, 4.8 Gy, and 2.4 Gy for the 1cm, 2cm, and 3cm bands, respectively. The estimated volume-average dose for the overall gradient field area (8-2 Gy) was 3.7 Gy [130].

For UI, the entire phantom was irradiated with a 30 cm x 30 cm field size for a total of 500 MU at a 600 MU/min dose rate with energy 6MV. The 30 cm size is large enough to cover the entire phantom. Each plate was irradiated for a uniform dose of 5.1 Gy. The control cell dish group followed the same protocol without receiving any irradiation.
Figure 3. Irradiation design. (A) Coronal slices of acrylic blocks with three cell plate holders; (B) Axial slices of acrylic blocks and solid water slabs (the red line indicates where the dose profiles to be taken); (C) Typical dose profiles of field 1 (orange), 2 (green), 3 (cyan), and the summation of the three fields (purple). Each plate was irradiated by three fields: Field 1, field size 1 cm x 1 cm, 500 MU, dose rate 600 cGy/min, energy 6MV; Field 2, field size 2 cm x 2 cm, 400 MU, dose rate 600 cGy/min, energy 6MV; Field 3, field size 5 cm x 5 cm, 200MU, dose rate 600 cGy/min, energy 6MV. Overlaps of the three fields generated a gradient dosage profile ranging from ~ 8 Gy at dish center to ~ 2 Gy at the edge of the irradiation dish; (D) A simplified diagram of three defined irradiation regions by their distances from the center of the plate: 1 cm circle, 2 cm circular band, and 3 cm circular band [130].
3.2.2 Extracellular ROS Detection after Irradiation and Cell Number Counting

The extracellular ROS formation was monitored by measuring the cytochrome c (Sigma-Aldrich Corporation, St. Louis, MO, USA) reduction at 2, 24 and 48h intervals following irradiation using Nanodrop 2000 spectrophotometer (Thermal Scientific, MA, USA) [132]. Cells were incubated with 5µM cytochrome c in cell culture medium [133]. Cytochrome c, when reduced by O$_2^{•−}$ (a major ROS), its absorbance at 550 nm is enhanced. The average absorbance at 540 nm and 560 nm was taken as the baseline and deducted from the absorbance peak at 550 nm to obtain the final reduction absorbance. This value is directly correlated to the concentration of extracellular O$_2^{•−}$ by an extinction coefficient of 18.5×10$^3$ M$^{-1}$cm$^{-1}$ [134]. To determine the average extracellular O$_2^{•−}$ production per cell, cell number in each cytochrome c-treated group was counted at 2, 24 and 48h after irradiation using Cellometer Mini (Nexcelom Bioscience, MA, USA), respectively. To determine whether the detecting signals are caused by extracellular O$_2^{•−}$ in our models, SOD (1500 U/mL, Sigma-Aldrich Corporation, St. Louis, MO, USA), a membrane-impermeable scavenger of extracellular O$_2^{•−}$, were applied following previous protocols [130, 132, 133].

3.2.3 Cell Viability Assay Using Trypan Blue

To confirm the cell viability results from Alamar Blue, the current study also used Trypan Blue staining (Life Technologies, Carlsbad, CA, USA) to quantify the average cell viability of each dish at 2, 24, and 48h after irradiation [135]. Cells were detached using trypsin (Life Technologies, Carlsbad, CA, USA) and re-suspended in DMEM. A 50
uL sample of cells was mixed with 0.4% Trypan Blue solution by 1:1. The cell viability was assessed and calculated using Cellometer Mini (Nexcelom Bioscience, Lawrence, MA, USA) [130].

3.2.4 Region-specific Cell Viability Assay Using Propidium Iodide (PI)

PI staining (Sigma-Aldrich Corporation, St. Louis, MO, USA) was used to examine the cell viability in the 1 cm circle, as well as the 2 and 3 cm bands of the irradiation area, providing more spatial information of the cell response to our designed irradiation intensities [10]. At 2, 24, and 48h after irradiation, cancer cells were incubated with 7.5 µM PI buffer for 15 min at 37 °C and then washed twice with PBS. PI fluorescence was monitored via Nikon Eclipse TS 100 microscope (Nikon Corporation, Tokyo, Japan). The setup for fluorescence imaging of PI fluorescence was as follows: Xenon lamp power (LPS-100, Photon Technology International, Inc. (PTI), Birmingham, NJ, USA); DeltaRAM X High-Speed Multi-Wavelength Illuminator LPS-100 (Photon Technology International, Inc. (PTI), Birmingham, NJ, USA); Nikon Eclipse TS 100 microscope and CCD camera (Nikon Corporation, Tokyo, Japan); ET excitation, 535±15 nm; ET emission, 617 ± 37.5nm, objective×20. The emitted signal was captured and presented as an image of 1392×1040 pixels on a computer monitor using Macro-ImageJ software (National Institutes of Health, Bethesda, MD, USA). Three random areas were selected from each band for the cell viability assay. The mean fluorescence was analyzed using Adobe Photoshop CS6 (64 Bit) software (Adobe Systems Inc., San Jose, CA, USA) to determine the cell viability [130].
3.2.5 Cell Apoptosis Assay Using Annexin V-Cy3

Annexin V-Cy3 (Abcam, Cambridge, United Kingdom) was employed to monitor cellular apoptosis levels after irradiation [136]. Cancer cells were loaded with ~30ug/ml Annexin V-Cy3 in a binding buffer (Abcam, Cambridge, United Kingdom) for 5 min in the dark at 2, 24, and 48h after irradiation. Annexin V-Cy3 fluorescence was detected via Nikon Eclipse TS 100 microscope (Nikon Corporation, Tokyo, Japan). The imaging setup was as follows: ET excitation, 543±15 nm; ET emission, 610±37.5; objective×20. The emitted signal was captured and recorded as an image of 1392×1040 pixels on a computer monitor using Macro-ImageJ software (National Institutes of Health, Bethesda, MD, USA). Three random areas were selected from each band for apoptosis evaluation of the band. The mean fluorescence intensity of each area was analyzed using Adobe Photoshop CS6 (64 Bit) software (Adobe Systems Inc., San Jose, CA, USA) to determine cell apoptosis levels [130].

3.2.6 Intracellular ROS Detection after Irradiation

The intracellular ROS production was monitored at 2, 24 and 48h after irradiation. Cancer cells were loaded with 5 µM dihydroethidium (DHE)/ethidium (ET) (Life Technologies, Carlsbad, CA, USA), a fluorescence probe primarily targeted for $O_2^-$, for 30 min at 37°C and then washed out with clean PBS (Life Technologies, Carlsbad, CA, USA). The setup for fluorescence imaging of ROS was as follows: ET excitation, 543±15 nm; ET emission, 610±37.5 nm, objective×20. The emitted signal was captured and presented as an image of 1392×1040 pixels on a computer monitor using Macro-ImageJ.
software (National Institutes of Health, Bethesda, MD, USA). Three random areas were selected from each typical band as a representative profile of ROS generation of that band. The mean fluorescence intensity of each area was analyzed using Adobe Photoshop CS6 (64 Bit) software (Adobe Systems Inc., San Jose, CA, USA). In order to determine the specificity of \( \text{O}_2^{-} \) probes (ET), we applied Tiron (0.25 mM, an intracellular scavenger of \( \text{O}_2^{-} \), Sigma-Aldrich Corporation, St. Louis, MO, USA) into the loading solution. These treatments would determine whether the detecting signals were caused by intracellular ROS or other chemicals released from our cell models [130, 133].

### 3.2.7 Statistical Analysis

Results are expressed as mean ± SE. Data were analyzed using one-way ANOVA with time, dosage and irradiation location. Statistical difference between various treatment groups were interpreted and displayed via Bonferroni post-hoc test using SPSS software (IBM, New York, US). \( p<0.05 \) was regarded as statistically different [130].

### 3.3. Results

#### 3.3.1 Extracellular ROS Formation was Higher in GI (8-2 Gy) than UI (5 Gy)

Extracellular ROS formation was monitored and represented by cytochrome \( c \) reduction as shown in Fig. 4A. Both UI (5 Gy) and GI (8-2 Gy) significantly stimulated ROS release when compared to the control group (0 Gy) at 2h (\( p<0.05 \) for both UI and GI), 24h (\( p<0.005 \) for both UI and GI), and 48h (\( p<0.001 \) for GI). SOD (1500 U/mL), a membrane-impermeable scavenger of extracellular \( \text{O}_2^{-} \), effectively diminished this
irradiation-induced ROS elevation. Furthermore, extracellular $\text{O}_2^{•−}$ levels demonstrate a significant increase from 2h to 24h after irradiation, followed by a decline at 48h for all three treatment groups (control, UI and GI). By comparing the UI and GI groups, we found that GI stimulated more ROS release as compared to UI at both 24h ($p<0.001$) and 48h ($p<0.001$), indicating a stronger redox-mediated bystander signal in the medium under GI [130].

### 3.3.2 Cell Viability was More Reduced by GI (8-2 Gy) than UI (5 Gy)

The average cell viability in each dish was evaluated at 2h, 24h, and 48h after irradiation by Trypan Blue. At 2h after irradiation, no difference in cell viability was observed between the irradiation groups (GI and UI) and control. Both UI (5 Gy) ($p<0.001$) and GI (8-2 Gy) ($p<0.001$) groups showed a decreased cell survival at 24h following irradiation compared to data at 2h (Fig. 5B). At 48h, irradiated cell viability was increased compared to data at 24h (UI: $p<0.05$; GI: $p<0.001$) which, however, were still largely lower than that of the control at 48h ($p<0.001$ for both UI and GI). GI groups (8-2 Gy) continue to display lower cell survival rate than that in UI (5 Gy) groups at both 24h ($p<0.001$) and 48h ($p<0.001$) after irradiation (Fig. 5) [130].
Figure 4. Rate of cytochrome c reduction at 2, 24 and 48h (n=6); 0 Gy ± SOD vs. 5 Gy ± SOD, and 8-2 Gy ± SOD. *p<0.05 vs. 0 Gy at the same time; †p<0.05 vs. 5 Gy at the same time; ‡p<0.05 vs. SOD groups at the same time and at the same dose; ††p<0.05 vs. 2h point at the same dose; †††p<0.05 vs. 24h point at the same dose [130].

Figure 5. Cell viability measured using Trypan Blue for 0 Gy, 5 Gy, and 8-2 Gy; Continued
Figure 5 Continued.
(measured from 6-9 randomly selected areas of ~300-900 cells on the Trypan Blue assay slides, respectively; mean ± SE). (A) Cell viability represented by a bar chart at 2, 24, and 48h after irradiation. (B) Cell viability represented by a line chart at 2, 24, and 48h after irradiation. *p<0.05 vs. 0 Gy at the same time; †p<0.05 vs. 5 Gy at the same time; ‡p<0.05 vs. 2h at the same dose; ††p<0.05 vs. 24h at the same dose [130].

Propidium iodide (PI), another cell death marker, was used to evaluate cell viability spatially at different regions of the dish (i.e., 1-3 cm bands) under both UI and GI. Based on the manufactory protocol, a higher intensity of PI fluorescence represents a lower cell viability (Fig. 6A and 6B). As shown in Fig. 6C, 6D, and 6E, irradiated cells (both UI and GI) emitted stronger fluorescence as compared to the control at 2h, 24h, and 48h (p<0.05). At 2h, the cell viability of GI (8-2 Gy) showed a decreased trend from a 1 cm circle to 3 cm band, which is consistent with the irradiation intensity profile of GI (8-2 Gy). Accordingly, the survival rate in the 3 cm band was significantly higher than that of the 2 cm band in GI (8-2 Gy) groups (p<0.01). Cell viability of both regions (2 and 3 cm) were higher than that of the 1 cm circle (p<0.005) in GI groups (Fig. 6C). It is noted that at 2h, UI (5 Gy) groups displayed lower cell survival rates than that of GI groups (8-2 Gy) in both 2 and 3 cm bands (p<0.01) (Fig. 6C). However, at 24h, the cell viability of GI (8-2 Gy) was decreased to a much lowered level than that of UI (5 Gy) in all the three regions (p<0.01 for 1 cm circle, p<0.01 for 2 cm band, and p<0.05 for 3 cm band). In addition, cell viability showed little difference between the three regions of GI (8-2 Gy) or UI (5 Gy) at 24h (Fig. 6D). At 48h after irradiation, GI (8-2 Gy)-treated cells still
displayed lower viability as compared to those under UI (5 Gy) in all three regions
($p<0.01$ for 1 cm circle, $p<0.05$ for 2 cm band, and $p<0.01$ for 3 cm band). No difference
of cell survival was observed among three regions of either GI (8-2 Gy) or UI (5 Gy) at
48h (Fig. 6E) [130].

Figure 6. Cell viability detected using PI fluorescence; Continued
Figure 6 continued.
(shown by red fluorescence; measured from three randomly selected areas of 300-500 cells in 1 cm circle, 2 and 3 cm bands, respectively); 0 Gy vs. 5 Gy vs. 8-2 Gy. (A) Representative image of high cell viability shown by PI fluorescence. (B) Representative image of low cell viability shown by PI fluorescence. (C) Grouped PI fluorescence measured from 1 cm circle, 2 and 3 cm bands at 2h after irradiation. (D) Grouped PI fluorescence measured from 1 cm circle, 2 and 3 cm bands at 24h after irradiation. (E) Grouped PI fluorescence measured from 1 cm circle, 2 and 3 cm bands at 48h after irradiation. Data were presented as mean ± SE; * p<0.05 vs. 0 Gy at the same circle/band; # p<0.05 vs. 5 Gy at the same circle/band; † p<0.05 vs. 1 cm circle at the same dose; ‡ p<0.05 vs. 2 cm band at the same dose [130].

3.3.3 Cellular Apoptosis was More Induced in GI (8-2 Gy) than UI (5 Gy)

As represented by Annexin V-Cy3 fluorescence, apoptosis occurred after irradiation in MCF-7 cells (Fig. 7). At 2h, all regions of UI (5 Gy), as well as the 1 cm circle and 2 cm band of GI (8-2 Gy), exhibited higher apoptotic levels than control. At this time point, the apoptotic levels were decreased from 1 cm circle to 2 cm band, as well as from 2 cm to 3 cm bands under GI (8-2 Gy), consistent with the intensity profile of GI (8-2 Gy) (Fig. 7C). In addition, apoptosis levels in 1 cm circle regions of GI (8-2 Gy) were higher than those of the 1 cm circle areas of UI (5 Gy) (p<0.05), while no significant difference was observed in 2 and 3 cm bands between GI and UI. At 24h, GI (8-2 Gy) showed higher apoptotic levels compared to UI (5 Gy) (p<0.05 in 2 cm band, Fig. 7D), while there was little difference in apoptotic levels among three regions of GI (8-2 Gy) (Fig. 7D). At 48h, Annexin fluorescence signals were stronger in the GI group (8-2 Gy) as compared to that in the UI (5 Gy) group (p<0.05 in 2 and 3 cm bands, Fig. 7E). There were no changes of apoptotic conditions in control groups (0 Gy) at 2h, 24h,
and 48h (Fig. 7F). Both UI (5 Gy)- and GI (8-2 Gy)-treated cells displayed higher apoptosis levels than the control group in all the three regions at both 24 and 48h as shown in Fig. 7D and 5E ($p<0.01$ for UI at both 24 and 48h; $p<0.001$ for GI at both 24 and 48h). Both UI (5 Gy)- and GI (8-2 Gy)-treated cells also showed increased levels of apoptosis at 24h ($p<0.05$), followed by a trend of decline at 48h in all three regions (Fig. 7G and 7H) [130].
Figure 7. Cell apoptosis at 2, 24, and 48h after irradiation. Cell apoptosis was detected using Annexin V-Cy3 shown by red fluorescence; measured from three randomly selected areas of 300-500 cells in 1 cm circle, 2 and 3 cm bands, respectively.

Continued
Figure 7 continued.

(A) Representative image of control cells stained with Annexin V-Cy3. (B) Representative image of apoptotic cells stained with Annexin V-Cy3; apoptotic cells emitted red fluorescence on membranes (arrows). (C) Grouped Annexin fluorescence in 1 cm circle, 2 and 3 cm bands at 2h after irradiation. (D) Grouped Annexin fluorescence in 1 cm circle, 2 and 3 cm bands at 24h after irradiation. (E) Grouped Annexin fluorescence in 1 cm circle, 2 and 3 cm bands at 48h after irradiation. (F) Grouped Annexin fluorescence in 1 cm circle, 2 and 3 cm bands of control. (G) Grouped Annexin fluorescence in 1 cm circle, 2 and 3 cm bands in UI (5 Gy). (H) Grouped Annexin fluorescence in 1 cm circle, 2 and 3 cm bands in GI (8-2 Gy). Grouped data were presented as mean ± SE; *$p<0.05$ vs. 0 Gy at the same irradiation circle/band; **$p<0.05$ vs. 5 Gy at the same irradiation circle/band; †$p<0.05$ vs. 1 cm circle at the same dose. ¶$p<0.05$ vs. 2h at the same circle/band; §§$p<0.05$ vs. 24h at the same circle/band [130].

### 3.3.4 Intracellular ROS Levels Following UI (5 Gy) and GI (8-2 Gy) Were Elevated in Different Patterns but Ended at Similar Levels

We evaluated the intracellular ROS generation by comparing the levels of $\text{O}_2^\bullet-$ formed at 2h (Fig.8), 24h (Fig.9) and 48h (Fig.10) after GI (8-2 Gy) and UI (5 Gy). Both irradiation treatment groups had significantly elevated $\text{O}_2^\bullet-$ levels, which were scavenged by Tiron. The $\text{O}_2^\bullet-$ generation at 2h after irradiation was represented by ET fluorescence in Fig. 8A. Quantified ROS fluorescence was summarized in Fig. 8B. For UI of 5 Gy treated group, there was no significant difference in $\text{O}_2^\bullet-$ formation across the 3 bands ($p = 0.337$). However, under GI (8-2 Gy), a marked reduction of $\text{O}_2^\bullet-$ formation was observed from regions of 1 cm circle to 3 cm band (Fig. 8B, $p<0.005$ for 1 cm vs. 3 cm and $p<0.05$ for 2 cm vs. 3 cm). The volume-average doses that were delivered to regions of 1 cm circle, 2 cm, and 3 cm bands were calculated as 7.3 Gy, 4.8 Gy, and 2.4 Gy, respectively. Notably, ROS production at the 2 cm band was significantly higher.
(p<0.05) in GI than UI cells (Fig. 8B) despite lower volume-average dose (4.8 Gy in GI vs. 5 Gy in UI) [130].

Figure 8. Intracellular ROS formation at 2h after irradiation detected by DHE/ET fluorescence; data were measured from three randomly selected areas of 300-500 cells in 1 cm circle, 2 and 3 cm bands, respectively; 0 Gy ± Tiron vs. 5 Gy ± Tiron, and 8-2 Gy ± Tiron; (A) Representative images of ROS formation (shown by red fluorescence); (B) Grouped ROS formation in 1 cm circle, 2 and 3 cm bands (mean ± SE); *p<0.05 vs. 0 Gy at the same circle/band; †p<0.05 vs. 5 Gy at the same circle/band; ‡p<0.05 vs. Tiron-treated group at the same circle/band and at the same dose; ψp<0.05 vs. 1 cm circle within the same treatment; ‡p<0.05 vs. 2 cm band within the same treatment [130].
Figure 9. Intracellular ROS formation at 24h after irradiation detected by DHE/ET fluorescence; data were measured from three randomly selected areas of 300-500 cells in 1 cm circle, 2 and 3 cm bands, respectively; 0 Gy ± Tiron vs. 5 Gy ± Tiron, and 8-2 Gy ± Tiron. (A) Representative images of ROS formation (shown by red fluorescence); (B) Grouped ROS formation in 1 cm circle, 2 and 3 cm bands (mean ± SE); *p<0.05 vs. 0 Gy at the same circle/band; †p<0.05 vs. 5 Gy at the same circle/band; †p<0.05 vs. Tiron-treated group at the same circle/band and at the same dose [130].
Figure 10. Intracellular ROS formation at 48h after irradiation detected by DHE/ET fluorescence; data were measured from three randomly selected areas of 300-500 cells in 1 cm circle, 2 and 3 cm bands, respectively; 0 Gy ± Tiron vs. 5 Gy ± Tiron, and 8-2 Gy ± Tiron. (A) Representative images of ROS formation (shown by red fluorescence); (B) Grouped ROS formation in 1 cm circle, 2 and 3 cm bands (mean ± SE); *p<0.05 vs. 0 Gy at the same circle/band; †p<0.05 vs. Tiron-treated group at the same circle/band and at the same dose [130].

At 24h, UI induced no significant difference in O$_2$•$^-$ formation across the three bands (p = 0.847) (Fig. 9). ROS production reached a similar level in 1 cm, 2 cm and 3
cm band, although different dosages were delivered across the bands in GI ($p = 0.167$, Fig. 9B). ROS formation at 48h after irradiation was summarized in Fig. 10. No significant differences in ROS production were detected across the three bands (1 cm, 2 cm and 3 cm) in the UI group ($p = 0.292$) or in the GI group ($p = 0.651$). Comparing the two irradiation groups (UI vs. GI), no significant differences in ROS were observed from the comparisons between the pairs of 1 cm, 2 cm, and 3 cm bands, respectively [130].

3.4 Discussion

Our study suggests that GI is superior to UI in both redox advantage and toxic dosage to surrounding tissues [137, 138]. Specifically, we have demonstrated that extracellular ROS were significantly increased following irradiation in both GI and UI (Fig. 4A). SOD-treated groups attenuated the IR-induced ROS generation which provided strong evidence that $O_2^-$ is the major type of ROS in our detection that was likely to transmit from irradiated cells to extracellular media. Previous research has shown that extracellular $O_2^-$ could directly mediate intercellular bystander effects [139, 140]. $O_2^-$ and other relatively stable ROS, such as $H_2O_2$ have been implicated in activation signaling pathways in bystander cells by regulating stress-related proteins such as JNK, ERK1/2, MAPK and p53 [141, 142]. Therefore, these observations suggest a potential mechanism underlying bystander effects by spreading “damage” to neighboring cells via an extracellular ROS-dependent pathway [130].

The trend of cell viability observed over the time is consistent with our extracellular ROS formation. As shown in Fig. 5 and Fig. 6, cell survivals were lower in
GI groups compared to control and UI groups at 24h, suggesting a more damaging effect induced by GI at 24h. Interestingly, the survival rate detected using Trypan Blue partially rebounded at 48h, possibly due to the cellular proliferation or repair mechanism of cancer cells (Fig. 5B). Notably, our band-specific cell viability data (Fig. 6) were highly consistent with the apoptosis analysis (Fig. 7), indicating irradiation-induced apoptosis may affect cancer cell viability following irradiation. This is consistent with a previous study, which proposed apoptosis as the potential mechanisms underlying bystander effects-related cell death [143]. Although both UI (5 Gy) and GI (8-2 Gy) groups showed an enhanced cell apoptosis and declining cell survival at 24h, the more cellular damaging effects (declined viability and enhanced apoptosis) were observed in the GI (8-2 Gy) group (Fig. 5-7). The irradiation dose levels at the 2 and 3 cm bands of GI (8-2 Gy) were similar (~2cm) or even lower (~3cm) than the corresponding regions of UI (5 Gy). However, the cancer-cell killing effects of GI (8-2 Gy) were more significant than that in UI (5 Gy) at all three regions at 24h and 48h. These observations may be attribute to the bystander effects in the 2 and 3 cm bands of GI (8-2 Gy), mediated by triggering the cellular apoptosis pathways in the lower-dosage regions [130].

Irradiation stress is known to boost biological ROS production, acting as a secondary messenger by propagating pro-inflammatory signals or causing oxidative damage [6, 144, 145]. Intracellular ROS generation has been implicated in mediating cellular apoptosis [9, 146]. To examine the redox scheme of bystander effects, the intracellular ROS formation, regarded as an index of oxidative stress, was studied in different gradient irradiated regions. As shown in Fig. 8, we found that higher doses
resulted in larger ROS generation across the 3 bands in GI (8-2 Gy) at 2h after irradiation. Our hypothesis is that oxidative stress manifested in the area under higher irradiation dose “migrated” to the region of lower irradiation dose. This hypothesis can be supported by the observation that there were no significant differences in intracellular ROS levels between the 3 bands in GI at 48h after irradiation (Fig. 8) [130].

Our result also suggests a potential advantage of GI therapy over UI in reducing the damage to neighboring healthy cells. In the current experiment, the outer circular band of GI receives a dosage of only ~2 Gy as compared to 5 Gy in the outer band of UI region. Accordingly, GI would cause less damage to the neighboring cells due to lower marginal dosage profile in clinical practice. Furthermore, ROS (O2•−) are mostly impermeable to cell membranes due to its polarity [92]. Other evidence also showed that the intracellular ROS migration could be independent of anion channels on the membrane [147]. It is speculated that extra- and intra-cellular ROS originate from different sources due to a distinct production pattern over time in this study (Fig. 4 vs. Fig. 11) [130].

The current study examines the bystander effects in response to GI of 8-2 Gy within 48h following radiation; future research may extend the monitoring time and also investigate cellular responses under a wider range of irradiation doses. In the future study, we will further our understanding on redox roles in the bystander effect. For example, we will perform a series of key experiments; as such we will include a control group in which SOD will be applied to the cell culture dish in order to block extracellular ROS signals after irradiation. The cell killing effects in this group will be evaluated in comparison to non-SOD treated cells to further confirm the roles of ROS in mediating
bystander effects. While it is essential to develop strategies to maximize the damaging effect in tumor cells and minimize the damaging effect in normal cells, understanding biological molecular mechanisms of bystander effects induced by radiation (e.g., cytokine signaling, redox regulation) can provide valuable insights into existing and future cancer radiotherapy.

Figure 11. Grouped data showing intracellular ROS formation at 2, 24 and 48 h after irradiation under GI (A) and UI (B) across the three regions (measured from three randomly selected areas of 300–500 cells in 1 cm circle, 2 and 3 cm bands, respectively; mean ± SE). *p < 0.05 vs. 1 cm circle at the same time; ψp < 0.05 vs. 2 cm band at the same time; †p < 0.05 vs. 2 h point at the same circle/band; ‡p < 0.05 vs. 24 h point at the same circle/band [130].

In conclusion, our study indicates a better therapeutic effect of GI (8-2 Gy) compared to UI (5 Gy) regarding lower cell viability and higher apoptosis. Higher levels of extracellular ROS production in GI may play a role in spreading bystander signals.
from high to low dose irradiation regions and mediating cell apoptosis. We suggest a potential advantage of GI over UI in achieving similar therapeutic effect while mitigating the damaging effect to neighboring healthy cells. More studies are in need to explore the molecular mechanisms of bystander effects in response to non-uniform irradiation. Other complementary techniques may be employed to confirm the effectiveness of GI in tumor treatment for the reduction of radiation toxicity [130].
Chapter 4 NADH in Hypoxic Preconditioning (HPC)

4.1 Introduction

Similar to I/R injuries that occur in cardiac muscle, skeletal muscles may suffer significant damage when exposed to hypoxic conditions and subsequent reoxygenation [148, 149]. Although proper levels of ROS are essential for muscle contraction, hypoxia can induce excessive ROS formation compromising the muscle function [150-152]. Such hypoxia-mediated injuries are commonly found in intensely-exercised skeletal muscles as well as the diaphragms of patients with respiratory diseases (e.g., COPD and asthma) [16, 149, 153, 154]. HPC, which repeatedly expose the muscles to a hypoxic environment for a short duration, has been found to effectively reduce muscle injuries observed during the subsequent hypoxia/reoxygenation [98, 153].

Multiple mechanisms have been proposed to explain the protective effects provided by HPC, in which ROS are likely to play critical roles [98]. Specifically, HPC has been shown to stabilize HIF-1α by triggering ROS (H$_2$O$_2$) formation. The activation of HIF-1α cascade enhances muscle adaptation to lower O$_2$ levels via the upregulation of the antioxidants expression and the stimulation of angiogenesis (Fig. 12) [153]. This is consistent with our previous observation that HPC treatment diminished elevated ROS signals during hypoxia [151]. Moreover, HPC-induced mitochondrial ROS production
presumably activates the PKC/ phosphoinositide 3-kinase (PI3-K)/ ERK signaling pathways, which ultimately inhibit the opening of mPTP [98]. mPTP opening is thought to be a key mechanism to cause cell death during reperfusion and reoxygenation periods [98, 155]. Although ROS are important in HPC protection, the current understanding of the HPC effects is still limited.

Nox located on sarcoplasmic reticulum (SR) is one of the primary sources of ROS in skeletal muscle [152]. NADH provides electron pools to reduce O$_2$ to O$_2$$^\cdot$ [4, 156]. Since mitochondria catalyze the transfer of H$^+$ and electrons from NADH to O$_2$ to generate ATP and water (Fig.12), O$_2$ concentration is linked with the levels of NADH in the cell [157, 158]. Low O$_2$ levels may hinder the mitochondrial respiratory efficiency, resulting in limited ATP availability and a large accumulation of NADH [159]. Chance et al. has measured NADH fluorescence in frog sartorius muscle in response to twitches, and observed a markedly enhanced signal during muscle contractions induced by the depletion of O$_2$ [160]. Elevated NADH levels have been implicated in Ca$^{2+}$ release via inositol 1, 4, 5-triphosphate receptors (IP$_3$R) activation. This pathway may account for increased cellular Ca$^{2+}$ levels due to hypoxia [161, 162]. Considering the potential involvement of NADH in ROS formation and Ca$^{2+}$ regulation, the characterization of NADH changes may provide insights into plausible preconditioning mechanisms. NADH can emit fluorescence at 420-480 nm when excited at ~320-380 nm; this critical optical property has become a useful tool for monitoring intracellular NADH levels [158]. Accordingly, we have developed the following approach to study the NADH fluctuations
within the mouse diaphragm throughout the HPC as well as the subsequent hypoxia/reoxygenation treatment.

Figure 12. A simplified schematic illustrating the mechanisms of $\text{O}_2^-$ production, NADH and $\text{Ca}^{2+}$ regulation under hypoxic condition as well as the potential HPC pathways. SOD, superoxide dismutase; HPC, hypoxic preconditioning; HIF-1α, hypoxia-inducible factor-1α; TCA cycle, citric acid cycle [153, 156-159, 161, 163].
4.2 *Ex Vivo* Evaluation of NADH in Diaphragm

4.2.1 Muscle Preparation and Tissue Chamber

C57BL6 mice were anesthetized via intraperitoneal injection with ketamine (70 mg/kg) and xylazine (10 mg/kg). After complete anesthesia, the diaphragm was quickly isolated from the mouse and preserved in Ringer’s solution (in mM: 21 NaHCO₃, 1.0 MgCl₂, 1.2 Na₂HPO₄, 0.9 Na₂SO₄, 2.0 CaCl₂, 5.9 KCl, 121 NaCl, and 11.5 mM glucose) bubbled with 95% O₂ and 5% CO₂. Muscle strips were dissected out and fixed in a glass bottom chamber (Model 800MS; Danish Myo Technology, Aarhus, Denmark; Fig. 13A) [164].

The muscle strip was superfused with Ringer’s solution from a 300 mL buffer reservoir using two pumps: one was used to transport the solution from the reservoir to the tissue chamber at a rate of ~0.2 mL/s and the other pump was used to return the chamber solution back to the buffer reservoir at a same rate (Fig. 13A). There are two separate solution sources bubbled either with 95% O₂-5% CO₂ for oxygenation or with 95% N₂-5% CO₂ for hypoxia. The superfusate was preheated using a heating rod regulated by an automatic temperature controller (Model#: TC-324, Warner Instruments Inc., Hamden, CT, USA). A mini thermometer connected with the temperature controller was placed in the chamber for real-time temperature monitoring (Fig. 13A). This two-pump system is important to maintain a stable temperature (~35-37 °C) as well as consistent O₂ levels in the superfusate for the muscle function.
4.2.2 HPC Treatment and Photometer System

Following an approximately 20-30 min rest in O₂-bubbled superfusate, the HPC-treated muscles were switched to a low-O₂ exposure for 2 min, immediately followed by a high-O₂ exposure for another 2 min. This cycle was performed five times by repeatedly switching the superfusate between the normal and hypoxic buffer sources. Following the HPC treatment, muscles were superfused with N₂-bubbled solution (PO₂: 40 Torr) for 30 min, which was followed by 15-min reoxygenation (PO₂: 550 Torr). Control groups followed the same procedure in the absence of HPC. NADH fluorescence (excitation: 360 ± 10 nm; emission: 460 nm) was recorded in real time during the whole process. As shown in Fig. 13B, the emitted fluorescence was captured via a photometer (Model#: D-104, Photon Technology International, Birmingham, NJ, USA) and then converted to digital signals (five points/s), presented using Felix GX 4.1.2 software on computer (Photon Technology International, Birmingham, NJ, USA).

Figure 13. Chamber design.
Figure 13 continued.

(A) Diaphragm muscle strip was loaded at the bottom of the chamber and superfused with the buffer using a two-pump circulation system; a thermometer is placed in the chamber to monitor the temperature in real time. (B) A circular, optically transparent window (1.4 cm in diameter) is located at the bottom of the chamber to allow the excitation light to illuminate the muscle and the emission light to go through and be captured by a photometer.

4.3 NADH Fluctuations during HPC and Subsequent Hypoxia

As shown in Fig. 14, our photometer setup demonstrates significant sensitivity to the changes of muscular NADH in response to O$_2$ fluctuations. The data are normalized by the baseline fluorescence, which is defined as the average fluorescence measured from a signal stable time interval before HPC. As expected, we observed a significant fluorescence increase following hypoxic exposure; and the signals returned to approximately the baseline level during the reoxygenation period. Previous studies have shown that the primary NADH fluorescence detected in the cell is the mitochondria NADH but not from the cytoplasm [158]. Therefore, the NADH signals in our observation are most likely a reflection of mitochondrial NADH changes. In addition, we found that NADH was kept at a high level throughout the 30-min hypoxia (Fig. 14). Elevated NADH/NAD$^+$ levels have been suggested to link with O$_2$•$^-$ generation in the mitochondria [16]. Therefore, the accumulation of NADH and other reducing molecules due to the low O$_2$ availability may account for the hypoxia-induced ROS overproduction in the diaphragm [149].
Figure 14. Time-course NADH fluorescence profile in diaphragm during HPC and the subsequent hypoxia/reoxygenation treatment. Data are presented as percentage of baseline fluorescence. HPC, hypoxic preconditioning.

ROS at a high concentration can induce large amounts of Ca\textsuperscript{2+} release from SR, which potentially causes muscle injuries [98, 163]. However, an HPC-mediated moderate Ca\textsuperscript{2+} increase may be beneficial to cells during exposure to low O\textsubscript{2} environment [162]. Bickler et al. has attributed the protective effects of preconditioning to augmented Ca\textsuperscript{2+} release induced by HPC. The study preconditioned neurons using a five-min hypoxic exposure and then observed increased NADH and Ca\textsuperscript{2+} release at the end of HPC. They suggested that the Ca\textsuperscript{2+} increase was triggered by the elevation of cytosolic NADH via IP\textsubscript{3}R activation. However, this study did not clarify the roles of mitochondrial NADH in the Ca\textsuperscript{2+} release process [162]. In the current experiment setup, we observed slightly decreased NADH signals after HPC treatment, indicating a small redox shift towards oxidative state in the diaphragm following preconditioning (Fig. 14). The discrepancy between our observation and Bickeler et al.’s findings may be linked with the differences
in our HPC protocols, detection methods, study conditions, and experimental tissues. Additional experiments are needed for a further elucidation of the potential interaction among NADH, ROS formation and Ca\textsuperscript{2+} release in hypoxic muscles associated with HPC.
Chapter 5 Conclusions

ROS play a universal role in biological system with their concentrations modulated by antioxidant defense. At low levels, ROS act as critical cellular signaling messengers; yet the over-accumulation of ROS can be induced under pathophysiological conditions, which are implicated in various pathological processes such as asthma and I/R injuries. In the current thesis, we mainly focused on two ROS-related treatments. First, we focused on the roles of ROS in radiation-induced bystander effects. In our observation, GI (8-2 Gy) led to more ROS release and a lower cell survival rate compared to UI (5 Gy). The results suggest an enhanced therapeutic efficiency of GI than UI in breast cancer cells but with less radiation toxicity on neighboring tissues. Significantly elevated ROS observed in GI-irradiated cells may act as signaling molecules to induce apoptosis in bystander cells under low-dose irradiation. However, additional studies are in need to optimize the GI doses, unravel the bystander mechanisms and establish GI mathematical models for its clinical application. Second, we have established a photometer system to monitor the levels of an important biological redox marker, NADH, in HPC-treated diaphragms. The data reflect significant alterations of NADH levels affected by O₂ availability. The HPC-induced redox shift in skeletal muscles may correlate with its protective effects in a low O₂ environment. In the future, this system
can be used to monitor the other important biological markers such as FAD, pH and Ca^{2+} in skeletal muscle with different treatments.
References


