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CYTOPROTECTIVE VERSUS NON PROTECTIVE AUTOPHAGY INDUCED BY
RADIATION IN HEAD AND NECK CANCER CELLS

A dissertation submitted in partial fulfillment of the requirements for the degree of Master of
Science at Virginia Commonwealth University

By

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LIST OF ABBREVIATIONS

IR Irradiation

DAPI 6- Diamidino 2- Phenylindole

TUNEL Terminal Deoxynucleotidyl transferase dUTP nick end labeling

AO Acridine Orange

AVO Acidic Vesicular Organelle

CQ Chloroquine

FACS Fluorescence activated cell sorting

LC3 light chain microtubule

RFP-LC3 Red fluorescent protein – LC3

DMSO dimethyl sulfoxide

Abstract

CYTOPROTECTIVE VERSUS NON PROTECTIVE AUTOPHAGY INDUCED BY RADIATION IN HEAD AND NECK CANCER CELLS

By Duaa M Bakhshwin

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science,
at Virginia Commonwealth University.

Virginia Commonwealth University, 2014

Major Director: David Gewirtz, PhD

Professor, Department of Pharmacology and Toxicology

The primary treatment options for head and neck cancer are radiation therapy or surgery, or both combined; chemotherapy is often used as an additional, or adjuvant, treatment. Patients treated with radiotherapy are exposed to a high cumulative dose of radiation over a period of time and there is a 17-33% chance of recurrence. High cumulative doses of radiation, a long time course of treatment, side effects and the possibility of recurrence provide the rationale for developing approaches for radiation sensitization, which could be helpful to patients in decreasing the dose, duration of radiation, side effects, or the chance of recurrence.

Radiation induces autophagy, which is a catabolic process involving the degradation of the cell's own components to generate energy under conditions of stress. Autophagy can be cytoprotective helping the cell to survive during stress such as nutrient deprivation or it can be cytotoxic, leading the cell toward death. We investigated whether blocking autophagy by the use of the antimalarial drug, chloroquine, could sensitize head and neck cancer cells to radiation.

Studies were performed using the HN30 human head and neck cancer line (p53 wild type) derived from the pharynx as well as HN6 human cells (p53 mutant) derived from the base of the tongue. Cell viability was determined by cell counting and clonogenic survival assays, autophagy was monitored based on acridine orange staining accompanied by flow cytometry, while western blotting, DAPI and TUNEL staining and PI/annexin/FACS were utilized for determination and quantification of apoptosis. Senescence was monitored by beta-galactosidase staining/ FACS analysis.

Radiation alone produced a transient growth arrest followed by proliferative recovery in both the HN30 and HN6 cancer cells. Radiation also promoted autophagy in both cell lines. The combination of chloroquine with radiation inhibited autophagy and promoted apoptotic cell death and suppression of proliferative recovery for the HN30 cells, but had little effect on sensitivity to radiation and proliferative recovery in the HN6 cells.

The data suggest that autophagy induced by radiation serves a protective function in the HN30 cells and that a blockade to autophagy by chloroquine drives the cell toward apoptosis and death. In contrast, autophagy in HN6 cells appears to be non-protective as a pharmacological blockade did not sensitize the HN6 cells to radiation. These studies support the premise that autophagy induction by radiation need not necessarily have a cytoprotective function and further indicates that caution should be exercised in efforts to sensitize head and neck cancer to radiation through the clinical suppression of autophagy.

1.1 Introduction

Cancer is a term used for diseases in which abnormal cells divide without control and are able to invade other tissues. Metastatic tumor cells can also spread by traveling through the blood stream and the lymphatic system and settling in different organ sites. However, not all cancers are malignant and invade other tissues as some may be benign and do not invade or metastasize. (cancer.org)

Cancerous cells lose their growth regulatory controls in large part due to DNA damage and the accumulation of mutations in some genes, especially those responsible for cell growth and division; the genes that promote cellular growth and are called oncogenes (such as c-myc and RAS) while the genes that ordinarily inhibit cellular growth are the tumor suppressor genes such as p53 and Rb (Bai L et al 2006,).

There are many different types of cancer, depending on the tissue from which they are derived, and the tumor cells often behave differently in their rate of growth and the response to treatment.

1.2 Epidemiology of Head and Neck Cancer

Half of all men and one-third of all women in the US will develop cancer during their lifetimes. (cancer.org) and according to the National Cancer Institute, head and neck cancer accounts for 3 to 5 percent of all cancer in the United States. (Jemal A et al, 2010)

These types of cancer are more common in men and in people older than age 50. The most recent estimates for head and neck cancers in the United States are for 2014 is about 37,000 people will

get head and neck cancer and an estimated 7,300 people will die of these cancers., Also this type of cancer is the 7th most common cancer worldwide (6th among men, 90% of which is squamous cell carcinomas (HNSCC).(American Cancer Society 2012).

1.3 Head and neck cancers are cancer that arises in the head or neck region (in the nasal cavity, sinuses, lips, mouth, salivary glands, throat, or larynx). (cancer.gov)

As with many types of cancer, there are risk factors that may increase the chances of developing head and neck cancer such as poor diet, and vitamin deficiencies. One of the most important factors that is likely to be responsible for most cases of head and neck cancer is smoking or tobacco use. According to the National Cancer Institute 85 percent patient with head and neck cancer have a history of tobacco use (Gandini S et al, 2008). Other important factors include alcohol consumption (Hashibe M et al,2006) human papillomavirus (HPV) infection, and exposure to radiation (Hashibe M et al, 2009). The Epstein-Barr virus (EBV) may be associated with the development of certain cancers (Chien YC et al, 2001) including nasopharyngeal cancer. Two inherited genetic syndromes, Fanconi anemia and Dyskeratosis congenita, may greatly increase the likelihood of developing throat and mouth cancers in people at an early age(Hashibe M et al, 2009)

Typical symptoms of head and neck cancers may include: a persistent swelling (for example, in the mouth) that does not heal, a persistent sore throat, difficulty swallowing (dysphagia), a change or hoarseness in the voice, a lump in the nose, neck or throat , unexplained weight loss, frequent coughing , ear pain or trouble hearing, and/or headache.(cancer.gov)

Most head and neck cancers are called squamous cell carcinomas as mentioned previously, because the surface layer of the head or neck region is formed of cells that are flat and squamous.

When the cancer is limited to this layer of cells, it is called carcinoma in situ. When the malignant cells spread into other layers of cells, it is called invasive squamous cell carcinoma. There are several types of head and neck cancer, classified according to the part of the body in which they occur, specifically: **(i)Laryngeal cancer:** arise in the larynx which is the voice box **(ii)Hypopharyngeal cancer:** The hypopharynx is the lower part of the throat, which surrounds the larynx. This part of the body is also called the gullet. **(iii)Nasal cavity and paranasal sinus cancer:** Behind the nose is a space where air passes on its way to the throat. This region is called the nasal cavity. The air-filled areas surrounding the nasal cavity are the paranasal sinuses. **(iv)Nasopharyngeal cancer:** The nasopharynx is an air passage located at the very upper part of the throat, just behind the nose. **(v) Salivary gland cancer:** The salivary glands make saliva, which is essential for breaking down food. **(vi) Oral and oropharyngeal cancer:** Both the mouth and the tongue are part of the oral cavity. The oropharynx is the middle of the throat, extending from the tonsils to the top of the larynx.(cancer.gov)

1.4 Treatment

There are several options available for the treatment of head and neck cancer, which include surgical removal of part or the whole tumor depending on the place and the stage of the tumor. Other options include the use of chemotherapy in which doctors administer anti-cancerous medications which have the ability to kill cancer cells. Some of the chemotherapies approved by FDA for the treatment of head and neck cancer are: Methotrexate, Fluorouracil, Bleomycin, Cetuximab, and Cisplatin.

One of the best and most frequently utilized options for head and neck cancer is radiation therapy, which involves exposing the tumor to a high energy beam which leads to killing or stopping the

tumor growth. Often, radiation and surgery treatments are used together. As the focus of this thesis project is radiation therapy of head and neck cancer, this will require a more detailed discussion of this topic. Rayotta Bando et al.

1.5 Radiation Therapy;

Radiation therapy works directly to damage DNA or by creating reactive oxygen species which will make different types of damage to the DNA (Yokoi, K., et al, 2005). Double strand breaks tend to be the most harmful form of DNA damage (Yokoi, K., et al, 2005), which can lead to growth arrest or cell death by the activation of various signaling pathways. The doses used to treat patients with head and neck cancer generally ranges between 66-74 Gy (2.0 Gy/fraction; daily Monday-Friday in 7wk). Although radiation is a very effective treatment for head and neck cancer, there is always some chance of recurrence (up to 30%) that may be due to tumor cells that survive through disruptions in cell death (primarily apoptotic) pathways,(Masuda and Kamiya 2012). Consequently, use of a radio sensitizing agent could help to reduce the dose or duration of the treatment and increase the survival.

Because radiation is also likely to harm normal cells around the tumor, many side effects may occur. Some side effects could subside after a few days or weeks while others could be permanent. These include: mouth ulcers (feels like small cuts or sores in the mouth), dry mouth (xerostomia), difficulty in swallowing, alterations in taste or smell, voice changes, stiffness of the jaw and bone decay, skin alteration, and fatigue. One of the worst side effects that may be permanent is dry mouth (xerostomia), due to damage of the salivary glands.(cancer.gov)

Radiation will affect cells in many ways by harming the DNA and different responses to radiation may include: apoptosis, senescence, and autophagy.

1.6 Apoptosis

Apoptosis is a form of programmed cell death which is highly important for physiological processes. It is one of the most important protective mechanisms for the health of the living tissues and to maintain the number of normal cells in that system (Elmore, 2007). Apoptosis occurs normally in embryogenesis and aging (Fulda & Debatin et al, 2006), as a defense mechanism such as in immune reactions or when cells are damaged by disease or noxious agents and this is considered pathological apoptosis. Cells that have certain limited life span and turn over, such as red blood cells or immune cells undergo physiological apoptosis (Majno et al ,1995). Stresses, DNA damage, radiation or chemotherapy also can activate apoptosis (Fulda & Debatin et al, 2006).

When apoptosis is activated, morphological changes occur which could include cellular shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (Potten et al, 2001). Apoptosis is unlike necrosis, which is considered traumatic cell death that results from acute cellular injury where cells will enlarge and swell with karyolysis (Majno G et al,1995). Moreover, apoptosis is an energy-dependent cell death and very controlled processes, whereas necrosis is an energy-independent and uncontrolled process that can be due to a nonspecific injury to the cell membrane (Majno G et al,1995).

Apoptosis can be activated and initiated either by intrinsic or extrinsic pathways. Both pathways lead to the activation of a family of aspartic acid-specific cysteine proteases, named caspases (Elmore, 2007). These caspases are the main components of apoptosis. Up to now at least 14 caspases have been discovered and at least 11 of them are in human cells (Shi, 2002). There are two types of caspases, initiators and effectors. Initiator caspases (2, 8, 9) are the ones responsible for cleaving and activation of the effector caspases (3,6,7) (Shi, 2002). Effector caspases will then activate different proteins leading to apoptosis; also effector caspases are capable of self-activation (Kaufmann et al, 1999).

The intrinsic apoptosis pathway is activated by factors inside the cells e.g. lethal DNA damage (Wang, 2001, Shi, 2002). A series of reactions take place that generally activate the p53 tumor suppressor protein, which will activate BAX and BID which are pro-apoptotic proteins. BAX and BID will then bind to the outer membrane of mitochondria and make pores in the mitochondria; eventually the proteins inside the mitochondria, such as cytochrome C will leak. Cytochrome C will bind to pro caspase 9, protease 9 will be activated leading to activation of other caspases (caspase cascade) and lead to proteolysis of other proteins and subsequent DNA fragmentation (figure 1.1) (Elmore,2007)

The extrinsic pathway is initiated by an extrinsic stimulus such as radiation or extracellular signals e.g. tumor necrosis factor α (TNF α) which will stimulate the tumor necrosis factor receptor (TNFR) family; in turn this will activate the adaptor molecule Fas-associated death

domain (FADD) and caspase 8 and then initiates apoptosis via the cleavage of caspase 3 or 7 (figure 1.1) (Hengartner 2000, Shi, 2002).

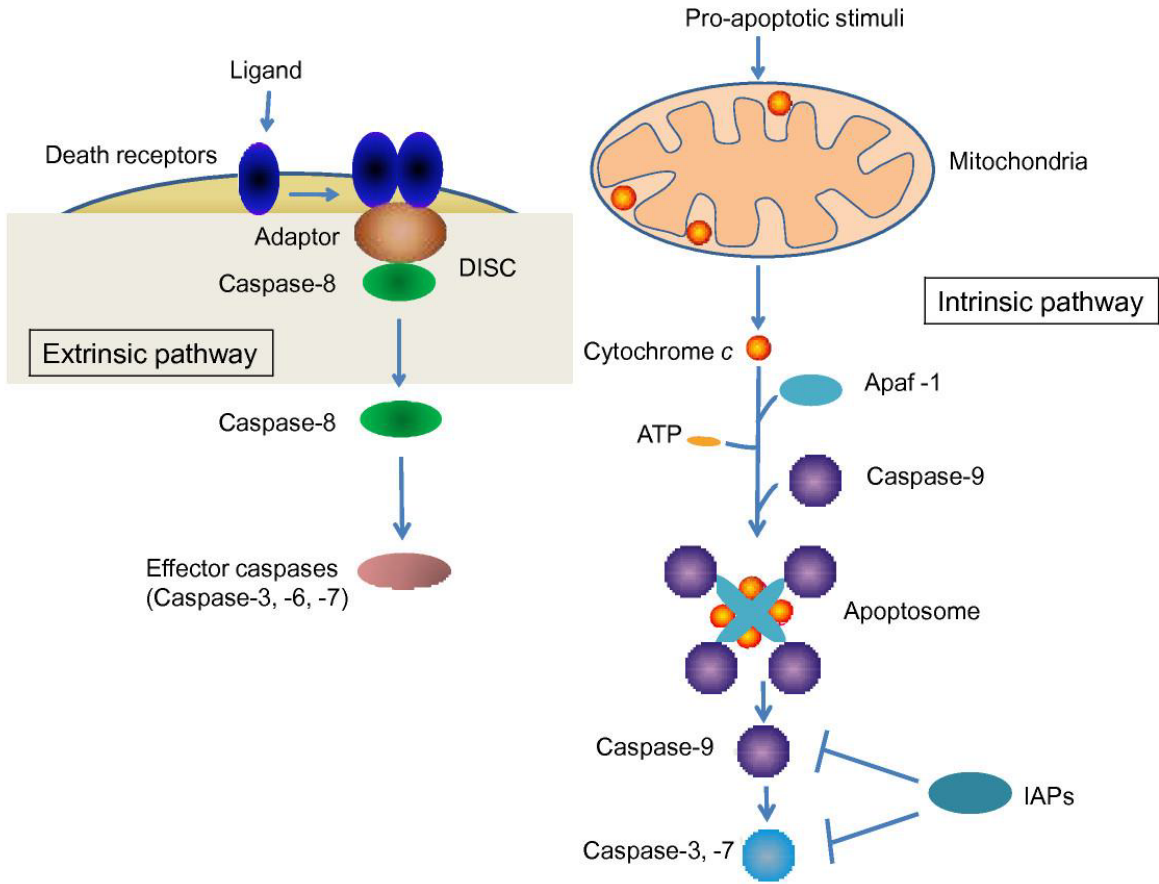


Figure 1.1 Extrinsic & Intrinsic Apoptotic Pathways (molecularbrain.com)

1.7 Senescence

Replicative senescence is considered to be an irreversible growth arrest of cells (Campisi., 2000). Senescent cells show resistance to growth signals and apoptotic pathways (Campisi., 2007) and they become flattened and enlarged with expression of β -galactosidase activity (Vigneron & Vousden, 2010). Senescence is induced by different stresses such as DNA damage, radiation or cytotoxic drugs (Ben-Porath & Weinberg, 2004). One of the forms of senescence can be induced by oncogenes and it is referred to as 'Oncogene induced senescence' (Serran & Collado, 2006). Irregular senescence is linked with the progress of some diseases such as atherosclerosis, osteoarthritis, muscular degeneration, ulcer formation, Alzheimer's dementia, diabetes, and immune exhaustion (Vergel M, 2011). Also defects in the senescence signaling pathway may lead to infinite growth of cells, which may lead to tumor formation. Thus, cellular senescence is assumed to be a tumor suppressing mechanism as it stops infinite cellular divisions.

Senescence growth arrest is controlled mainly by two tumor suppressor proteins, p53 and pRb. p53 is activated by stimuli such as radiation; other proteins such as ATM/ATR and Chk1/Chk2 are also activated (figure 1.2) (Ben- Porath, 2005). p21 is a very essential protein in this pathway, acting as a transcriptional target of p53 and as mediator of p53 induced senescence. The other pathway involves p16 and phosphorylation of Rb and E2F expression. Those two pathways work partially together but can also work independently as indicated in the review by (figure 1.2) (Campisi., 2007).

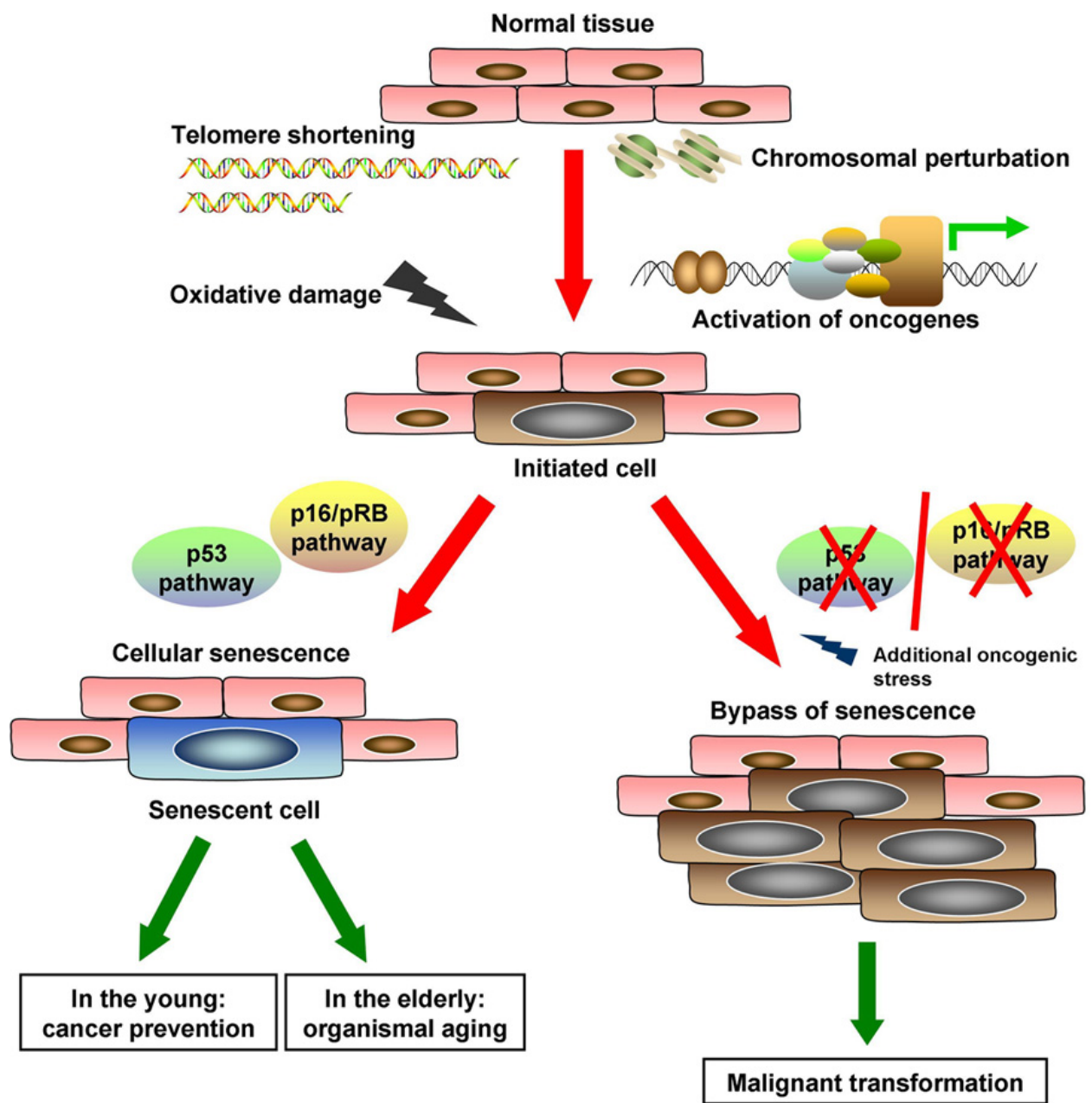


Figure 1.2: senescence pathway, *Frontiers in Bioscience* 14, 4044-4057, January 1, 2009

1.8 Autophagy

Autophagy (self –eating) was first described by Christian de Duve in 1963 as a basic catabolic mechanism in which cells break down un-necessary or damaged organelles and use them as a source of fuel during starvation or stress. (Chen N et al, 2009)) Autophagy is important in maintaining cellular homeostasis (Mizushima N, 2007). Autophagy begins with a formation of a double membrane vesicle, termed an autophagosome, which elongates, matures and fuses with a lysosome to degrade its contents by the aid of acidic hydrolase enzyme (figure 1.3) (Mizushima N, 2007). This process is referred to as macroautophagy, while microautophagy occurs when the lysosomes fuse directly around a cytosolic organelle and ingest it. Cellular stress could activate autophagy, for example DNA damage or starvation will inactivate mTOR which is one of the most important inhibitors of autophagy. Numerous autophagy related genes (ATGs) are essential for initiation and elongation of the autophagosome. Also many proteins have a significant role in autophagy including the light chain protein (LC3) which is necessary for stabilization of the autophagosome. Upon activation of autophagy, LC3 I is changed to LC3 II by the aid of ATG7 (figure 1.3)(Tanida, I et al 2008). p62 is an ubiquitin binding protein that is degraded when autophagy flux occurs, which will lead to a reduction of the level of p62 and indicate that autophagy has gone to completion. (Tanida, I D et al, 2008)

Autophagy is generally thought to be a cell survival mechanism. Autophagy is induced by radiation and other toxic agents (Koukourakis M et al, 2009). It can act as mode of cell death or as a resistance mechanism (Jin S, White E, 2007). Upregulation of autophagy can work as a tumor cell survival mechanism under conditions such as stress (Gewirtz et al 2009). On the other

hand, blocking autophagy to sensitize cancers may be an effective therapeutic strategy to overcome drug resistance (Koukourakis M et al, 2009)

There is a continuing debate as to whether autophagy is cytoprotective, cytotoxic or might be non-protective. When autophagy is protective and it is blocked by medication (for example: chloquine) or genetic silencing, the cells will die or go under growth arrest. In contrast, having cytotoxic autophagy will lead cells to die if induced, and blocking it will allow for cell survival. Non protective autophagy will not disturb cell growth if autophagy is blocked.

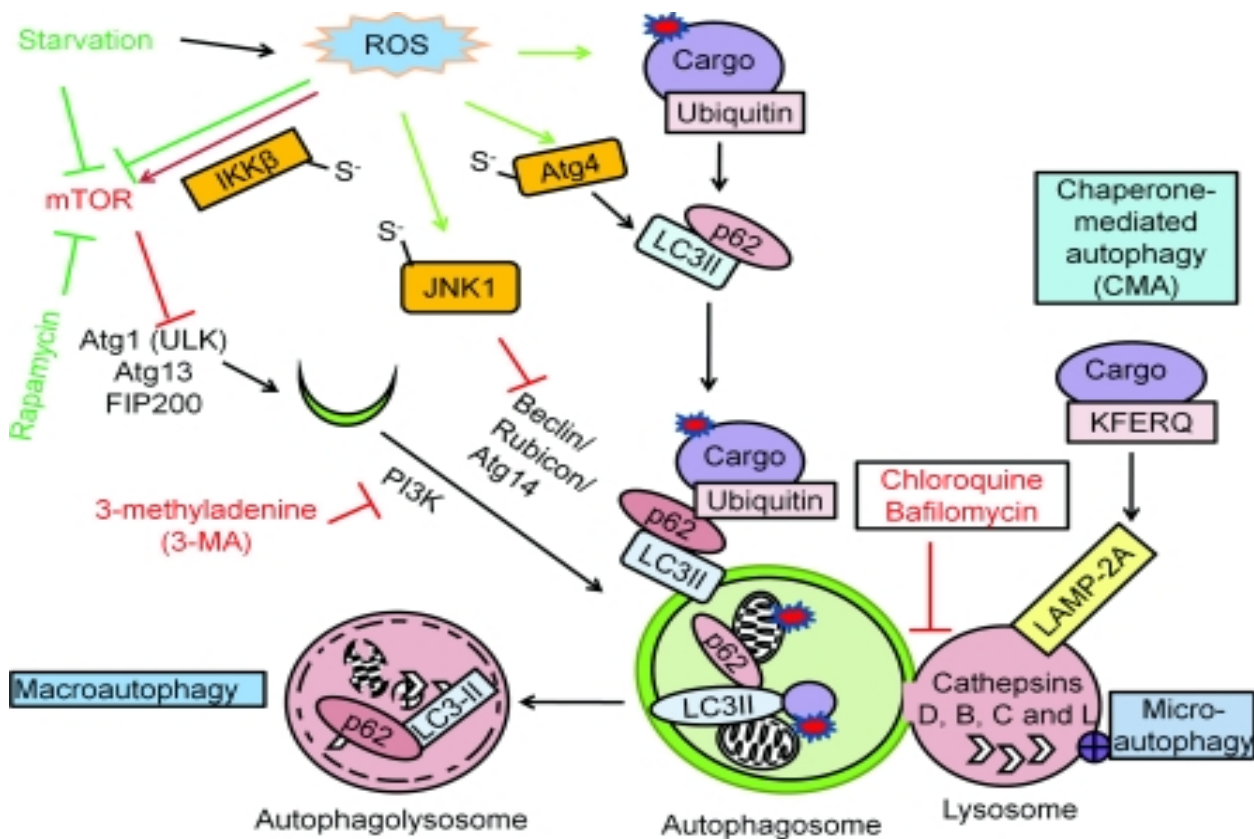


Figure 1.3 : autophagy pathway. Biochem. J. (2012) 441 (523–540).biochemj.org

1.9 Chloroquine

Chloroquine is a basic compound used as an antimalarial medications as well as in the treatment of autoimmune disease due to its mild suppression of the immune system. Chloroquine is a lysosomotropic agent that accumulates in the lysosomes of cells and inhibits the acidification of the lysosomes (Solomon and Lee, 2009). Chloroquine also inhibits the last step of autophagy by interfering with the fusion of the lysosome to the autophagosomes. The net result is the accumulation of undigested vesicles (Schneider P et al,1997) (Donohue E et al ,2013).

Material and Methods:

The p53 wild-type (WT) HN30 and p53 mutant HN6 Cells were obtained from Dr. Yeudall's laboratory and cultured in DMEM medium containing 10% FBS (GIBCO Life Technologies, Gaithersburg, MD) 2 mM L-glutamine and 1% of penicillin-streptomycin (GIBCO Life Technologies, Gaithersburg, MD). All cells were maintained at 37°C under a humidified, 5% CO₂ atmosphere. Cells were routinely sub cultured by trypsinization (0.25% trypsin, 0.03% EDTA, GIBCO) (incubation for 5-10 minutes) upon reaching confluence. All cell cultures were examined by microscope for bacterial and fungal contamination prior to experiments.

Additionally, all cell lines were determined to be free of mycoplasma.

Cells were exposed to γ -IR using a ¹³⁷Cs irradiator. Radiation treatment of 4 Gy radiation were administered after 4 hour of chloroquine treatment (5 μ M).

2.1 Drug preparation & Treatment

Chloroquine (CQ) was obtained from Sigma (St. Louise, MO). A stock solution was prepared by mixing CQ powder in water. A stock concentration of 50mM was prepared and kept at -20 °C.

2.2 Cell survival:

Cell viability was determined by Trypan blue exclusion at various time points after the last dose of radiation. Cells were harvested using trypsin, stained with 0.4% Trypan blue dye (Sigma, T8154), and counted using phase contrast microscopy; a minimum of three experimental replicates were conducted.

2.3 Clonogenic Survival assay

The ability of cells to form colonies was evaluated by plating 200 cells in triplicate in a 6 well tissue culture dishes for the following experimental conditions: control, CQ 5 μ M ,IR 4 GY, CQ+IR (CQ for 4hours then radiate the cells and wash CQ after 24 hours). Cells were permitted to adhere overnight. The next day, cells were treated with the indicated drugs or radiation for 24 hours, drug was removed and fresh media was added every other day. At day 9, cells were washed one time with 1X PBS before fixation with 100% of methanol for 10 minutes. Methanol was aspirated and colonies were stained with crystal violet dye (1%) in deionized water for 10 minutes. Colonies were counted visually in each well. Data were normalized relative to untreated controls, which were taken as 100% survival; a minimum of three experimental replicates were conducted

2.4 MTT Assay

The MTT assay was used to choose the effective dose of Chloroquine for additional experiments. For the MTT assay, cells were plated in 96 wells plate at a density of 5000 cells /well in 200 μ l of MEM Alpha medium. Cells were allowed to adhere to the plates overnight and then treated with different doses of Chloroquine. CQ doses used ranged from 2.5 μ M- 50 μ M. Cells were incubated with drug; then media was then aspirated and cells were incubated with the MTT solution (2mg/ml PBS) in each well for 3 hours at 37°C. The MTT solution was removed, 100 μ l of autoclaved DMSO was added and plates were shaken for 10 min. In the MTT assay, the MTT (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent is reduced by enzymes in living cells to formazan. DMSO acts as solubilizing solution dissolving insoluble purple formazan product into a colored solution.

2.5 Detection of autophagy in cells by staining with acridine orange

Acridine orange could be used as an indicator of autophagy, the volume of the cellular acidic compartment was visualized by Acridine orange staining .The cellular acidic compartment volume is increased in autophagy and therefore staining of the acidified autophagosome is used as an autophagy marker. (Paglin et al., 2001). 200,000 Cells were plated in 6-well plates, allowed to adhere overnight, and treated as described above for the cell viability study. At the day of the assay, cells were incubated with medium containing Acridine orange dye which was diluted in PBS in a ratio of 1:10000 (prepared in the dark) (Invitrogen, A3568) for 15 min; the Acridine orange was then removed, cells were washed once with PBS, fresh media was added, and fluorescent micrographs were taken using an Olympus inverted fluorescence microscope, SC 35 camera. Again, all images provided are at the same magnification (20X). The number of cells with increased acidic vesicular organelles was determined by counting at least three representative fields per treatment condition; a minimum of three replicate experiments were conducted.

2.6 FACS studies of Acridine Orange Staining to quantify Autophagy (acidic vacuole organelle AVO)

FACs studies were used to detect and quantify the AVO in HN30 and HN6 cells. Cells were plated, treated, and harvested in day 1,3,5, and 7 for FACS analysis .Then cells were suspended in 500µl PBS and stained with acridine orange stain in 1:10000 dilution in PBS for 10 min; data

were then analyzed using by BD FACS Canto II and BDS DIVA software. 10,000 cells were required within each gated regions.

2.7 DAPI staining Assay

One of the methods for detecting apoptosis is using DAPI (4',6-diamidino-2-phenylindole) fluorescent stain that binds firmly to DNA in A-T rich regions. It detects nuclear DNA fragmentation. Cells were collected and centrifuged at 1,500 rpm (G 18.0), for 5 minutes. Cells then were fixed on slides using Cytospin 4 (Shandon) and were stored at 4 °C until ready for staining. Cells were immersed in a 4% formaldehyde solution for 10 min at room temperature then washed twice with PBS for 5 min each. Slides were then submerged in 1:2 mixture of Glacial acetic acid: Ethanol for 5 min at room temperature. Again slides were washed with PBS twice. Slides were prepared with 10µl of a 1:1000 dilution of Vecta shield. Slides were covered with a coverslips and fixed by using clear nail polish and micrographs were taken using an Olympus inverted microscope SC 35 Type 12 camera. All images taken are at the same magnification 20X.

2.8 TUNEL Assay (Terminal deoxynucleotidyl transferase dUTP nick end labeling)

One of the methods for detecting apoptosis is by detecting DNA fragmentation through the use of Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). fluorescent

nucleotides are attached to the 3'-OH end of fragmented DNA with the help of the enzyme terminal transferase, which emits bright green fluorescence in apoptotic cells.

Detection Kit (Roche 03 333 566 001) was used for this assay. Cells were collected and centrifuged at 1,500 rpm . Slides with these cells were made by spinning cells at 10,000 rpm for 5 minutes in a Shandon Cytospin 4 (Thermal Electron Corp) and refrigerated at 4°C until used. For staining, cells were submerged the cells in a 4% formaldehyde solution for 10 min at room temperature then washed twice with PBS for 5 min each. Slides were then submerged in 1:2 mixture of Glacial acetic acid: Ethanol for 5 min at room temperature. Again slides were washed with PBS twice. A circle was drawn on each slide by ImmunoPen (CaliBiochem, San Diego, CA). 1mg/ml of Bovine Serum Albumin was added for 30 min at room temperature followed by two washes with PBS for 5 min each. An enzyme mix containing 4µl of 5X reaction buffer, 0.2µl of Terminal Transferase, 2µl of 25 mM COCl₂, 0.4µl of fluorescein-12-dUTP and 13.4µl water per sample were added and then cells were kept warm at 37°C for 1 hour. Slides were washed two times with PBS at room temperature, covered with a coverslips and fixed by using clear nail polish and micrographs were taken using an Olympus inverted microscope. All images taken are at the same magnification 20X.

2.9 FACS studies using Annexin V – PI staining to detect apoptotic and necrotic cells

FACs studies were used to detect and quantify the apoptotic and necrotic cells in HN30 and HN6 cells. Cells were plated, treated, and harvested in day 1,3,5, and 7 for FACS analysis. Then cells were labeled by using a FITC Annexin V fluorescence from apoptosis detection kit I (BD Biosciences Lot # 556547). Labeling was through adding 500µl of binding buffer, 5µl Annexin V- FITC and 5µl Propidium Iodide to each sample. Cells were mixed well and kept in the dark for 15 minutes. Cells which were labeled with Annexin V- PI were measured by Flow Cytometry

and analyzed by BD FACS Canto II and BDS DIVA software. 10,000 cells were required within each gated regions. Y axis was the PI and x axis was for Annexin V

2.10 β Galactosidase Staining Assay for Senescence

In senescent cells, the β galactosidase enzyme is upregulated and cleaves the X-Gal substrate. Cells were plated and treated as indicated in the cell viability assay. At several time points (cells were washed once with PBS, fixed with 2% formaldehyde and 0.2% Glutaraldehyde for 10 minutes followed by a wash with PBS. Cells were then incubated in a CO₂ free incubator with staining solution. A CO₂ free incubator is essential in order to maintain pH at 6. The staining solution or staining buffer for the beta galactosidase assay contains 20mg/ml X-Gal in dimethyl formaldehyde, 0.2M citric acid/Na phosphate buffer at pH 6, 100mM potassium ferrocyanide, 100mM Potassium ferricyanide, 5M NaCl, 1M MgCl₂ in distilled water. The senescence signal can be observed as early as 2 hours after incubation but for best results overnight incubation was preferred. Cells were washed with PBS and images were captured using light microscopy.

2. 11. Statistical analysis

Statistics were performed by using ANOVA followed by Tukey .The significance of group values was determined based on a p-value of $p < 0.05$

Results:

3.1 Radiation effect in cellular growth:

HN30 and HN6 cells were treated with different doses of radiation to determine the effects on cellular growth (not shown). 4Gy was chosen as the dose to be used for further studies since it promoted growth arrest followed by proliferative recovery in both tumor cell lines (although there was also some initial cell death in the HN6 cells). Fig 3.1 presents the time course for the effect of 4 Gy radiation on HN30 cell line versus the control which showed a growth inhibition followed by recovery while Fig 3.2 shows the effect of 4Gy of radiation on HN6 cellular growth which showed cell death followed by growth inhibition and then recovery. Possible causes of the limited response to radiation (i.e. proliferative recovery) in both cell lines might be: promotion of cytoprotective autophagy, DNA repair or suppression of free radical generation and reduced DNA damage.

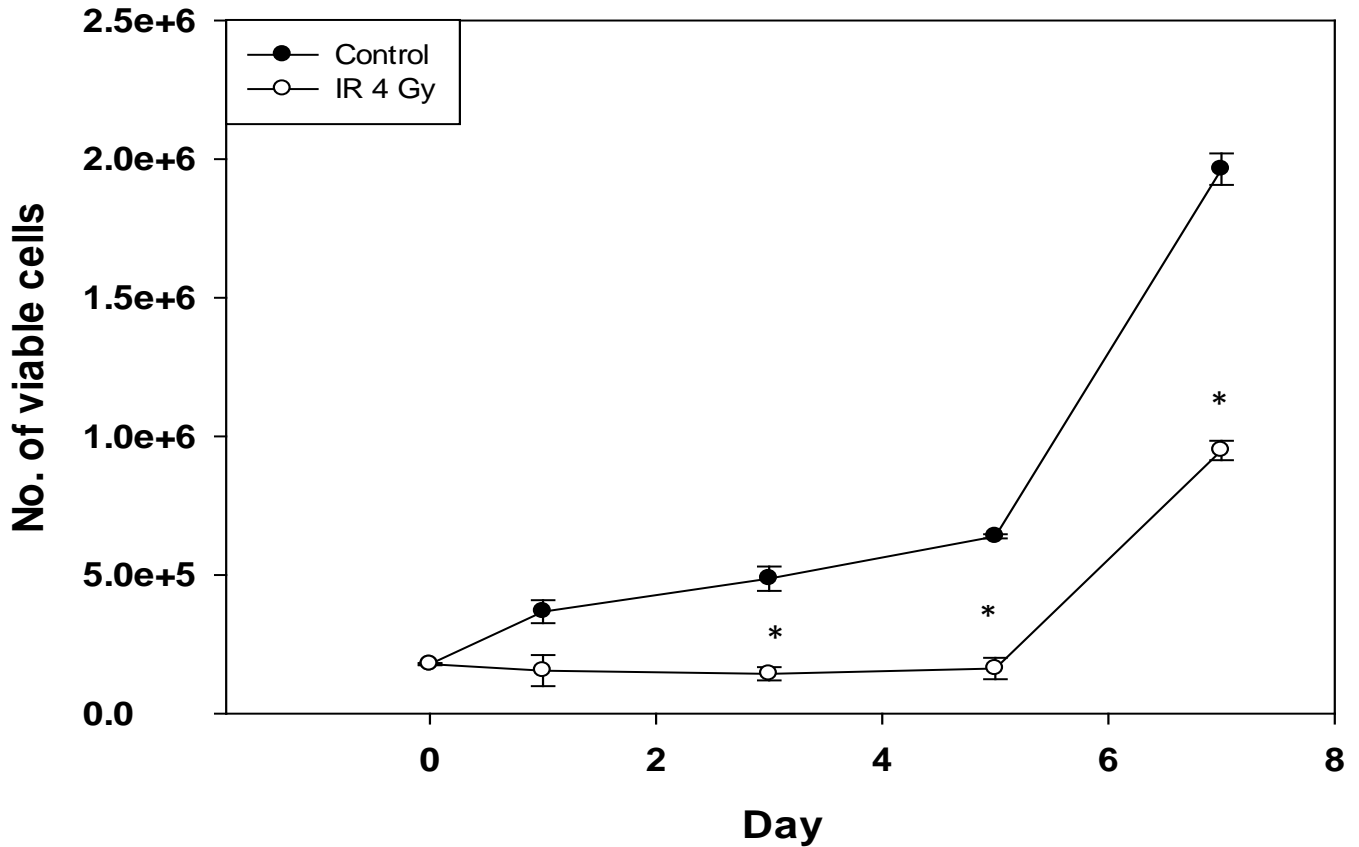


Figure 3.1 : Effect of radiation on viable cell Number(HN30 cells)

HN30 cells were plated in 6-well plates. Cells were plated in triplicate for each condition and viable cell numbers were determined by Trypan Blue Exclusion. Irradiation dose= 4 Gy. This figure is representative for one experiment .This experiment was performed at least three different times (* p<0.05 compared to control)

2D Graph 3

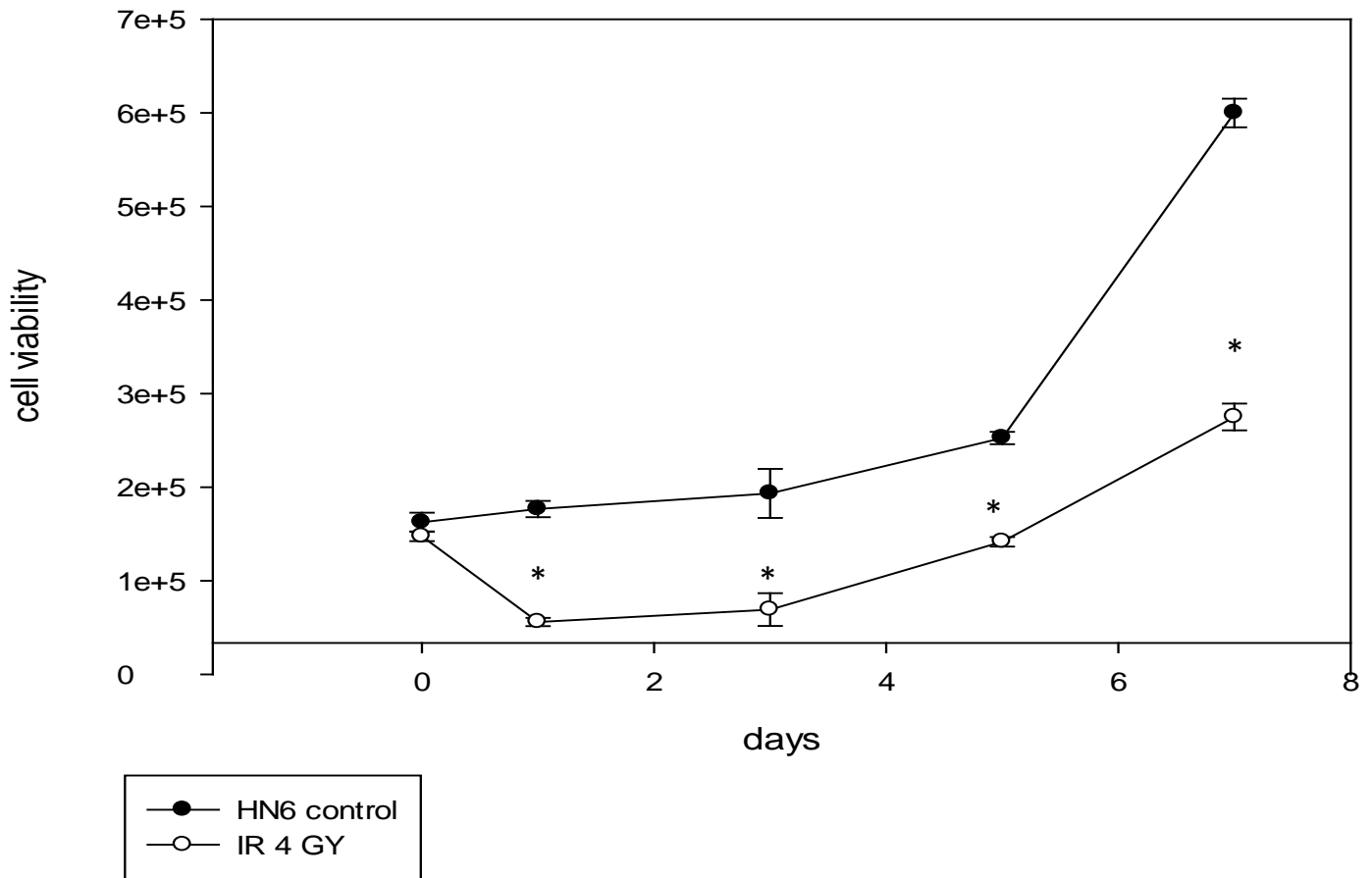


Figure 3.2: Effect of radiation on viable cell number (HN6 cells):

HN6 cells were plated in 6-well plates and viable cell numbers were determined by Trypan Blue Exclusion. Irradiation dose= 4 Gy. This figure is representative for one experiment .This experiment was performed at least three different times (* p<0.05 compared to control)

3.2 Radiation induces autophagy in HN30 and HN6 cell lines

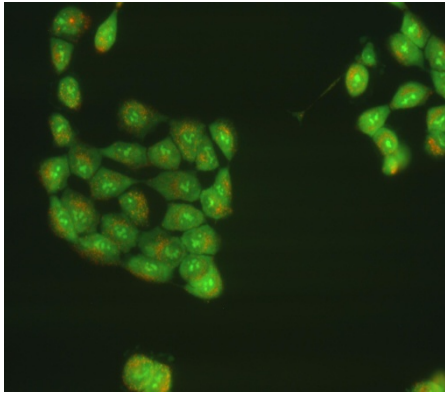
Radiation induces autophagy in many cell lines as shown in previous studies in our laboratory.

Upon radiation, MCF-7 and ZR-75 breast tumor cells undergo autophagy and upon inhibition of such autophagy, the cells are sensitized to radiation. (Bristol et al, 2012; Wilson et al,2012).

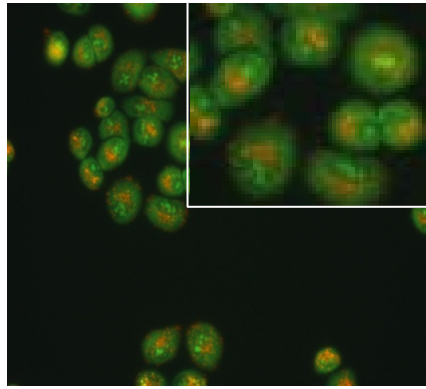
Acridine orange staining can be used to recognize cells having autophagy. Acridine orange is a lysomotropic dye that will accumulate in any acidic vacuole in a pH dependent manner. Most of the cells have acidic vacuoles but they are seen mostly in cells undergoing autophagy. At physiological pH, acridine orange give a green fluorescent color but when it is trapped within an acidic environment it becomes protonated, and emits bright red fluorescence (Klionsky et al, 2007).

In figure 3.3 A-B we evaluated the induction of autophagy using Acridine Orange staining to detect (acidic vesicular organelle AVO) or autophagosomes. Figures 3.3 A-B show the increase in the orange color vesicles which stains the autophagosomes in both cell lines and this means when cells are irradiated there is increase in AVO and autophagy. Also, there are some orange vacuoles in the control of both cell lines HN30 and HN6 that is indicative of basal autophagy.

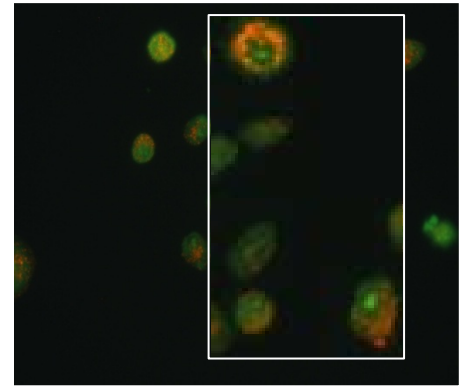
A



Control HN30

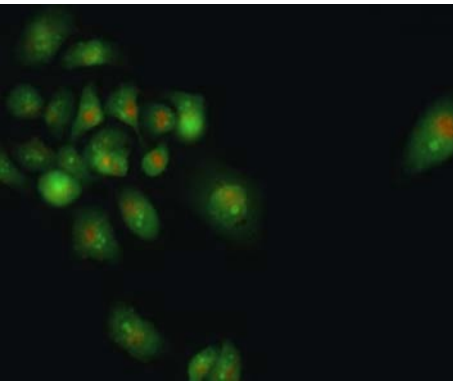


IR Day 1

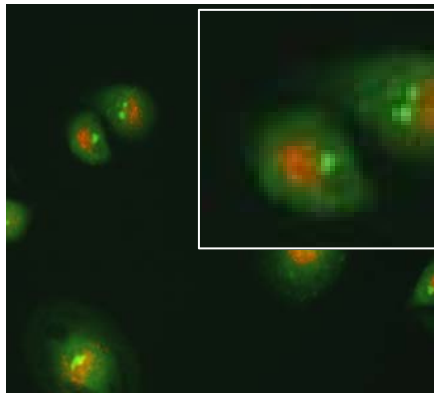


IR Day 3

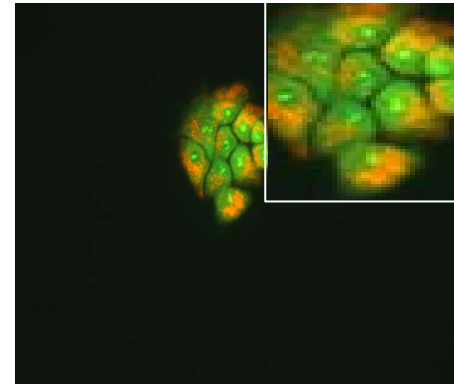
B



Control HN6



IR Day 1



IR Day 3

Figure 3.3(A-B): Induction of autophagy upon radiation:

Cell plated in triplicates in 6 well plates then exposed to 4 Gy radiation. Cells were then stained with Acridine Orange and observed under a fluorescent microscope (20X) on indicated days post radiation. A. for HN30 cells and B. for HN6 cells .An increase of the orange coloration indicate increased acidic vacuole formation within the cells upon exposure to radiation .Given images are representative of one of three experiments.

3.3 Inhibition of autophagy:

Choosing Chloroquine dose:

Chloroquine (CQ) has been used in many studies to sensitize cells to radiation (Bristol et al, 2012). In our laboratory autophagy inhibition sensitized breast cancer cells to radiation (Bristol et al, 2012, Wilson et al, 2012). Autophagy can be inhibited either pharmacologically or by genetic silencing of autophagy genes. (Rodemann et al, 2011). Chloroquine is a basic and lysomotropic drug that accumulates in the lysosome and interferes with the acidification of the lysosome (Solomon and Lee, 2009). It inhibits the last step of autophagy by preventing the lysosome fusion with the autophagosome and inhibits formation of the autophagolysosome . As a result the contents inside the autophagosome are not degraded and the autophagolysosomes accumulate inside the cell (Bursch et al., 1996).The MTT assay was used to decide the concentrations of CQ in order to use it as a pharmacological inhibitor of autophagy with limited toxicity. Cells were treated with CQ and were analyzed by measuring absorbance at 540nm. The MTT assay indicated that CQ concentrations of 2.5 μ M and 5 μ M are nontoxic. Thus, a 5 μ M dose (Figure 3.4) was selected for further treatments. Similar result has been seen in both cell line HN30 and HN6 so the dose of treatment for CQ was 5 μ M in both cell lines

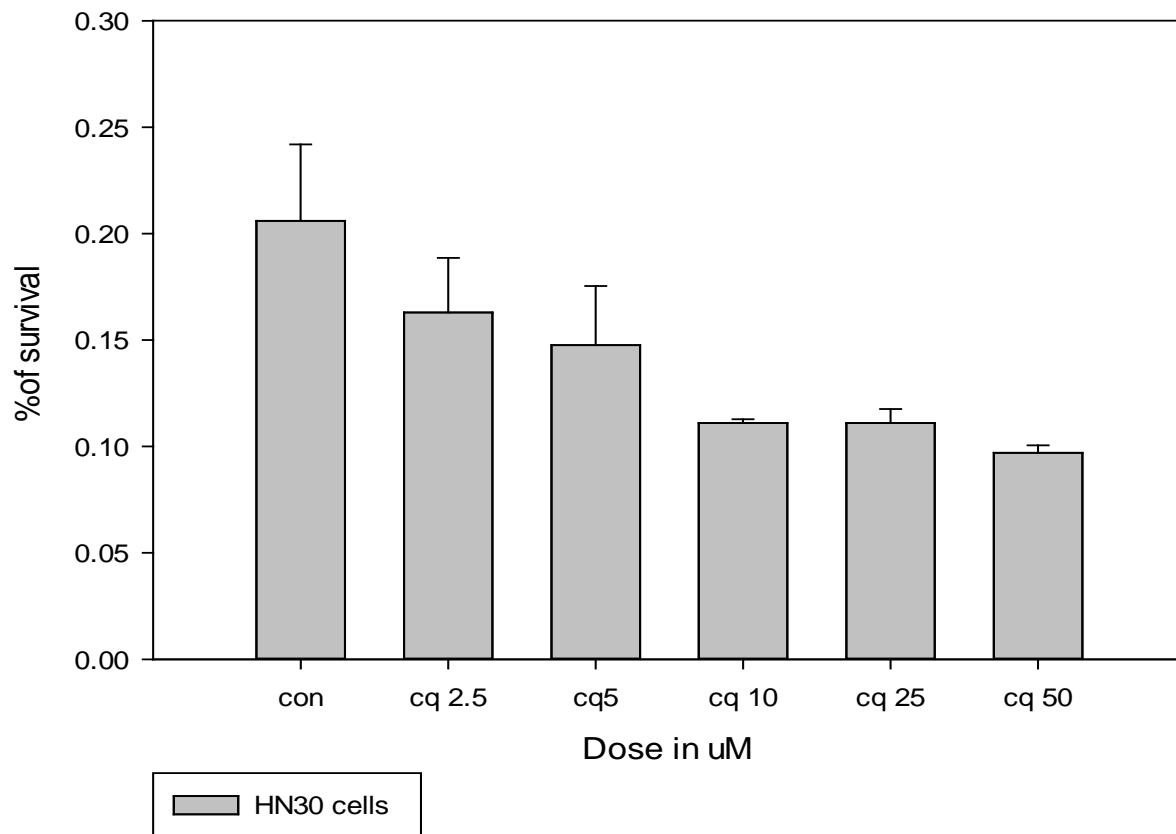


Figure 3.4: Determination of CQ dose

Viability of cells after treatment with CQ was measured using the MTT assay. Cells were plated in 96 well plates and treated with an increasing concentration of CQ. Then cell were incubated with the MTT solution (2mg/ml PBS) in each well for additional 3 hours, 100µl DMSO was used to dissolve the reduced reagent(purple in color) Formazan which is formed by the living cells. This experiment was repeated 3 different times

3.4 To determine whether Chloroquine will sensitize HN30 and HN6 tumor cells to radiation.

Figure (3.5-3.6) shows that treating cells with 5 μ M of chloroquine four hours prior to radiation increased sensitivity of HN30 to radiation. The cells undergo growth arrest with no recovery, which is different than the response that occurs with radiation alone. On the other hand, the HN6 cell line showed a similar pattern of growth and growth inhibition with radiation plus chloroquine as with radiation alone as the arrest phase was not evident in Figure 3.9-3.10 This was also confirmed by the clonogenic survival assay which is a highly sensitive assay used to check the ability of single cells to form colonies after radiation or chemotherapy treatment. a Figure 3.7 shows colony formation for HN30 cells. The upper panel shows cells treated with radiation only and the lower panel shows the IR+CQ treated cells. Figure 3.8 presents a quantification of the clonogenic survival assay showing 90% reduction of the number of colonies in cells treated with IR+CQ compared to control which indicates radiosensitization of the HN30 cells. In HN6 quantification of the clonogenic survival assay showed less than additive effect of both IR and CQ on the IRCQ treated cells figure 3.11, 3.12

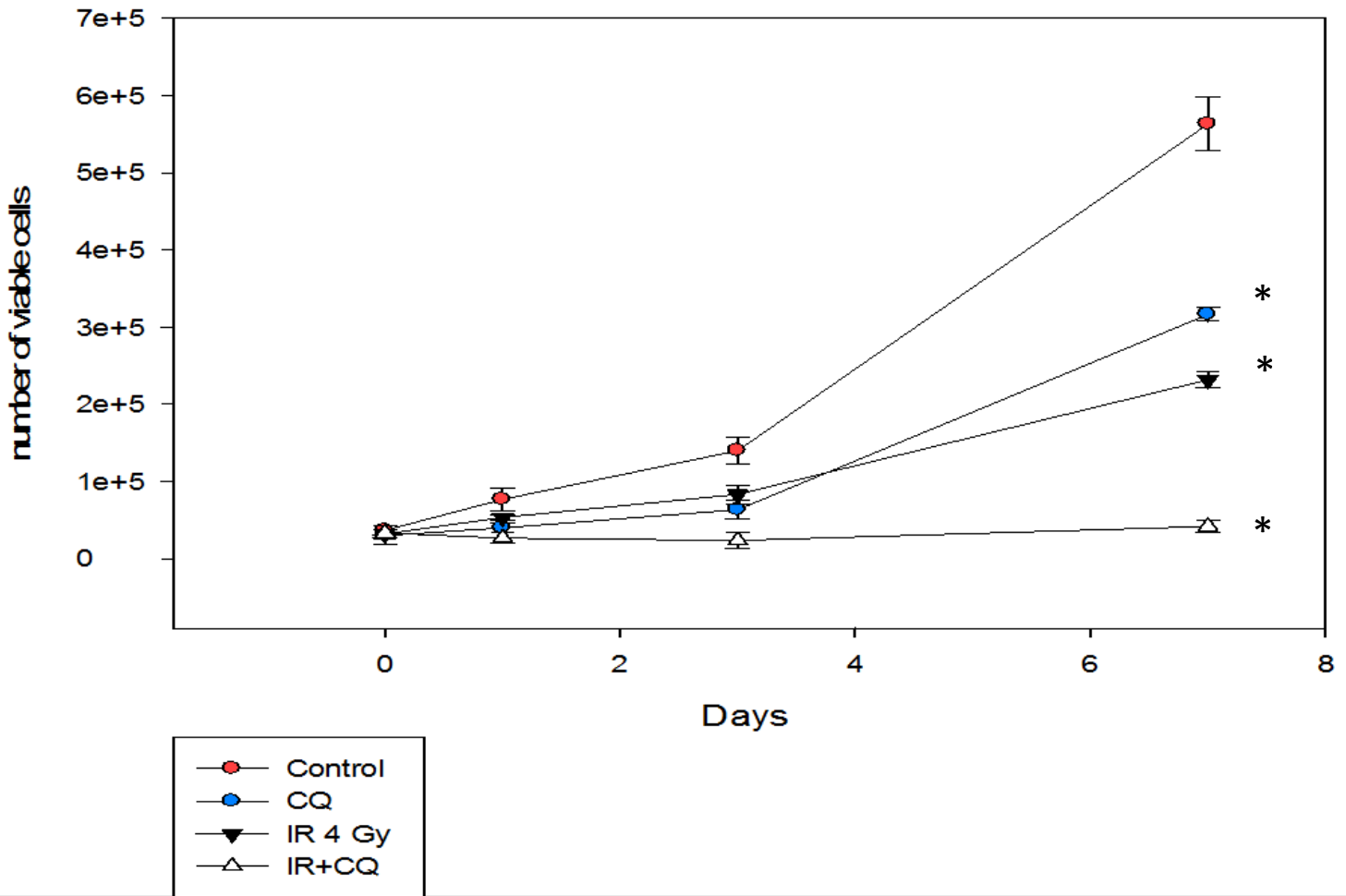


Figure 3.5: Analysis of the effect of inhibition of autophagy on radiation sensitivity using CQ in HN30 cells

HN30 cells were plated in 6-well plates each in triplicate and viable cells was measured by Trypan Blue exclusion. Radiation dose: 4Gy, CQ: 5 μ M. This figure is representative for one experiment .This experiment was repeated three times (* p<0.05 compared to control).

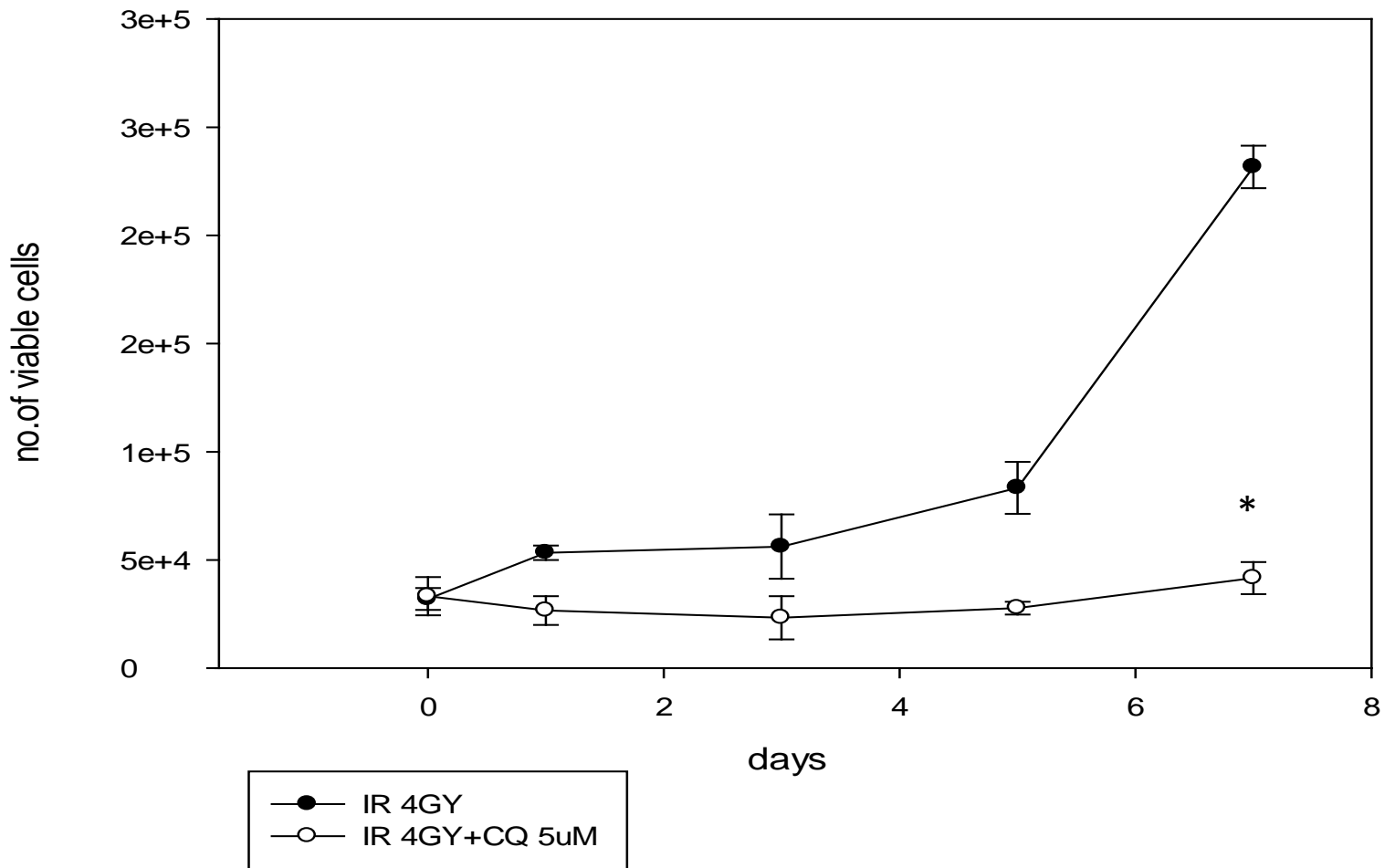


Figure 3.6: CQ sensitize HN30 cells to radiation

Time course for HN30 cells showing irradiated cells and the combined treatment IR+CQ.

Radiation dose: 4Gy, CQ: 5 μ m. This figure is representative for one experiment. This

experiment was performed 3 times (* p<0.05 compared to control)

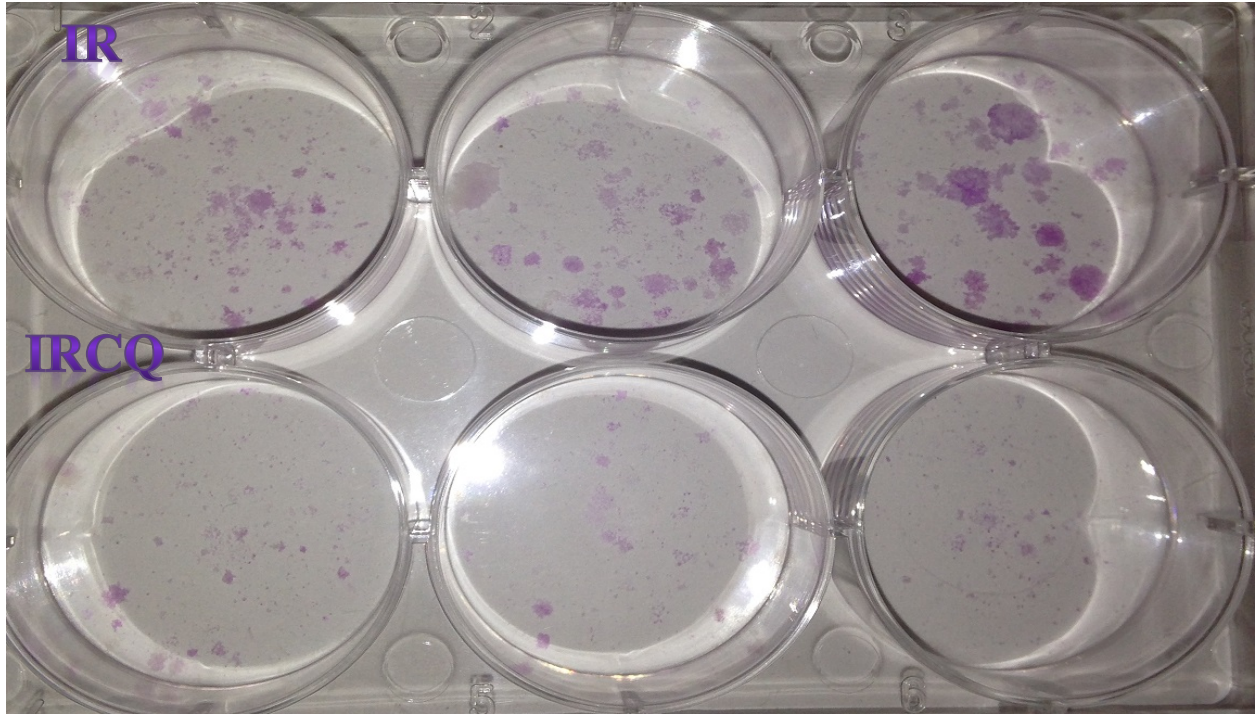


Figure 3.7: Clonogenic survival assay for HN30 cells

This figure shows HN30 cells exposed to IR alone and IR+CQ 2 weeks after treatment. Cells were plated in 6 well plates, each in triplicate. IR: 4 Gy , CQ:5 μ M , after 2 week cells were fixed and stained with crystal violet.

This experiment was repeated 3 different times

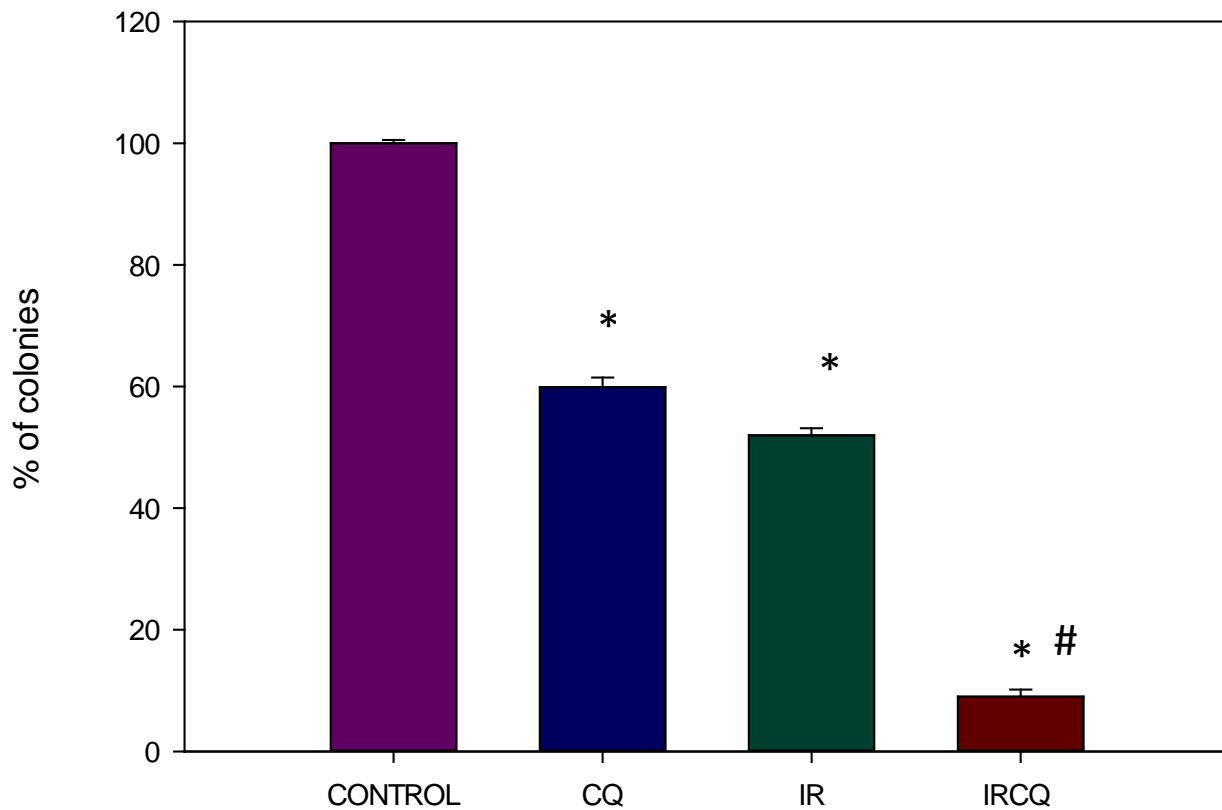


Figure 3.8: Quantification of Clonogenic survival assay for HN30 cells :

IR and CQ alone have similar effects on the number of colonies while IR+CQ decreased the colony formation by 90% .Experiment repeated at least 3 times. (* $p < 0.05$ compared to control, # $p < 0.05$ compared to IR)

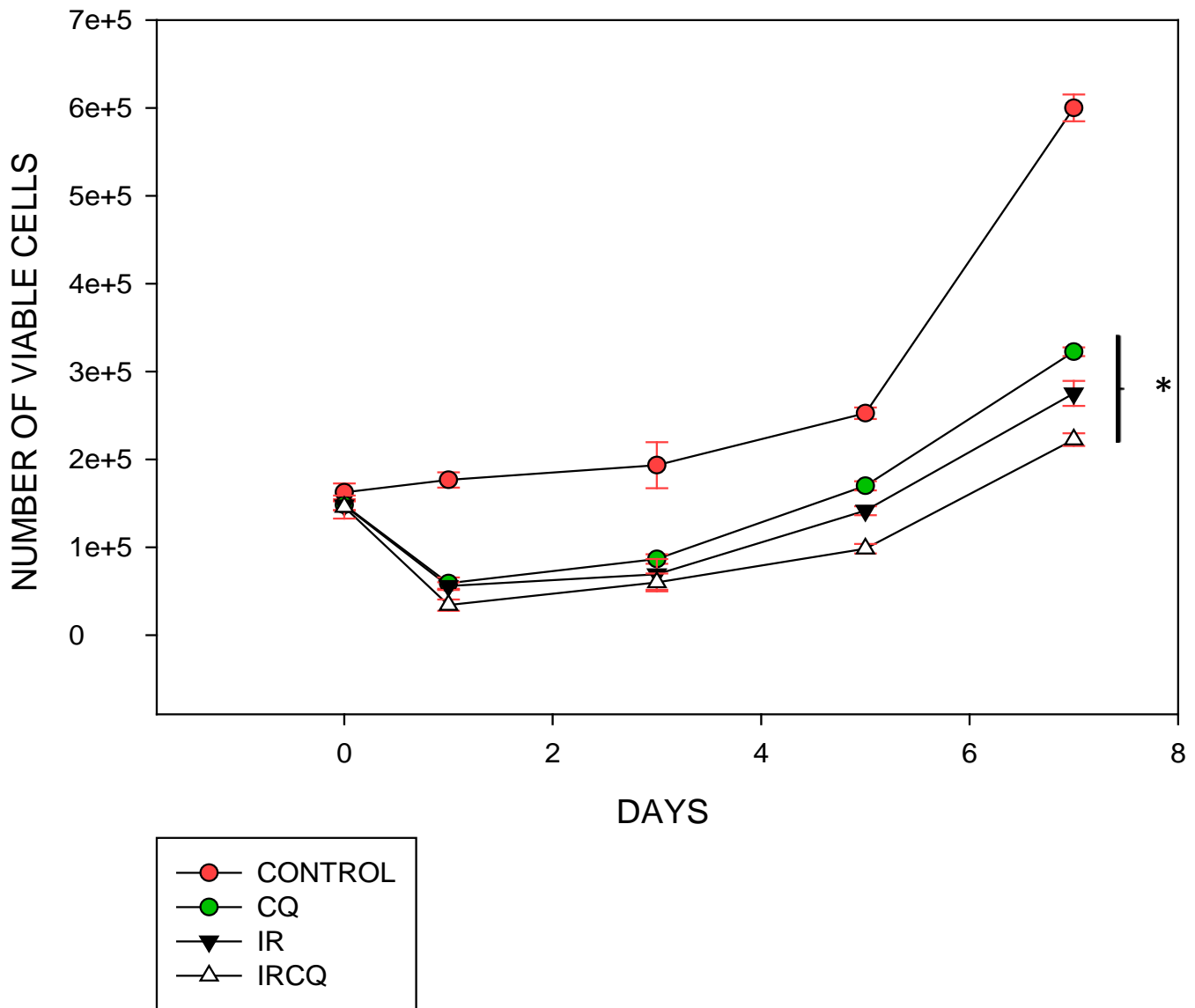


Figure 3.9: The effect of inhibition of autophagy on radiation sensitivity using CQ in HN6 cells: Time course for viable cells was measured by Trypan Blue exclusion. Radiation dose: 4Gy, CQ: 5 μ M. This figure is representative for one of the experiments. This experiment was performed 3 times (* $p < 0.05$ compared to control)

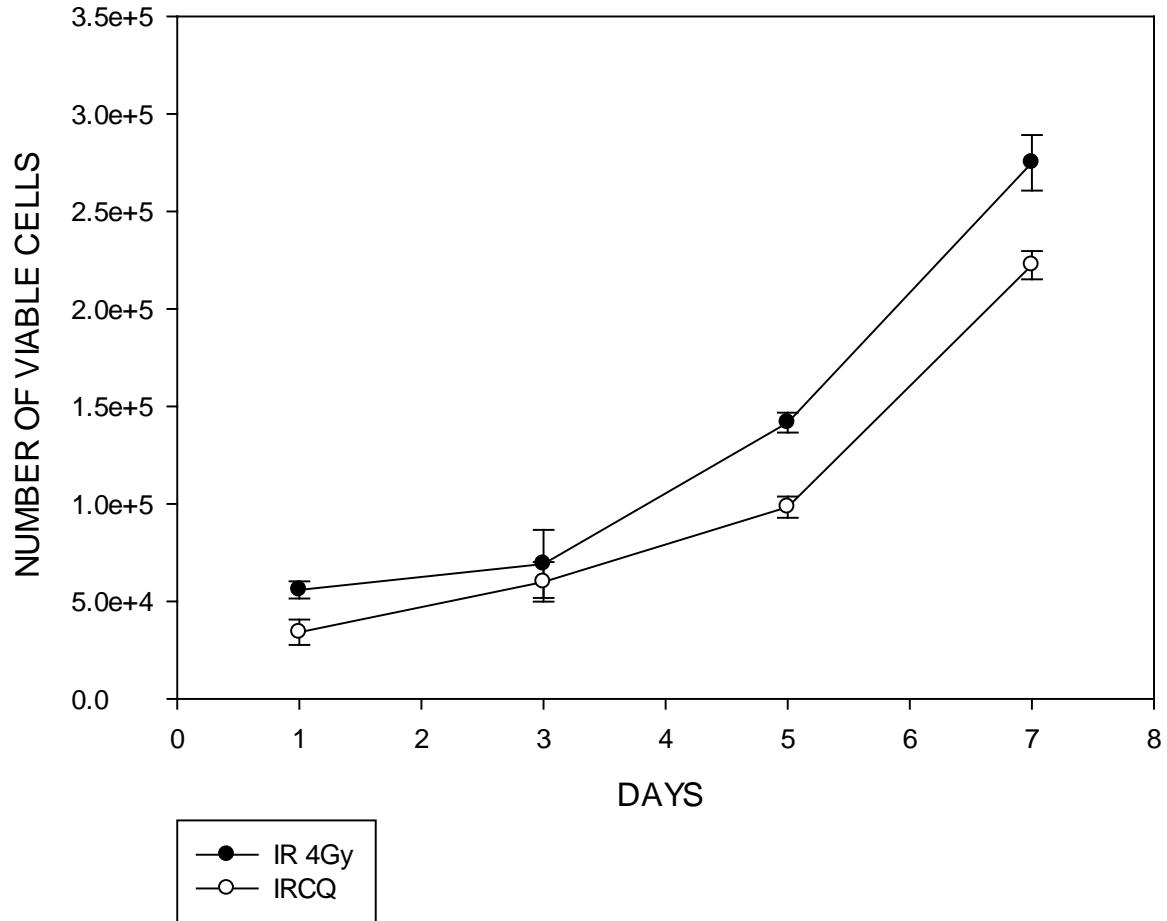


Figure 3.10: CQ fails to sensitize HN6 cells to radiation

Time course for HN6 cells showing irradiated cells and the combined treatment IR+CQ.

Radiation dose: 4Gy, CQ: 5 μ M. This figure is representative for one of the experiments. This experiment was repeated 3 different times. ($p > 0.05$ compared to control)

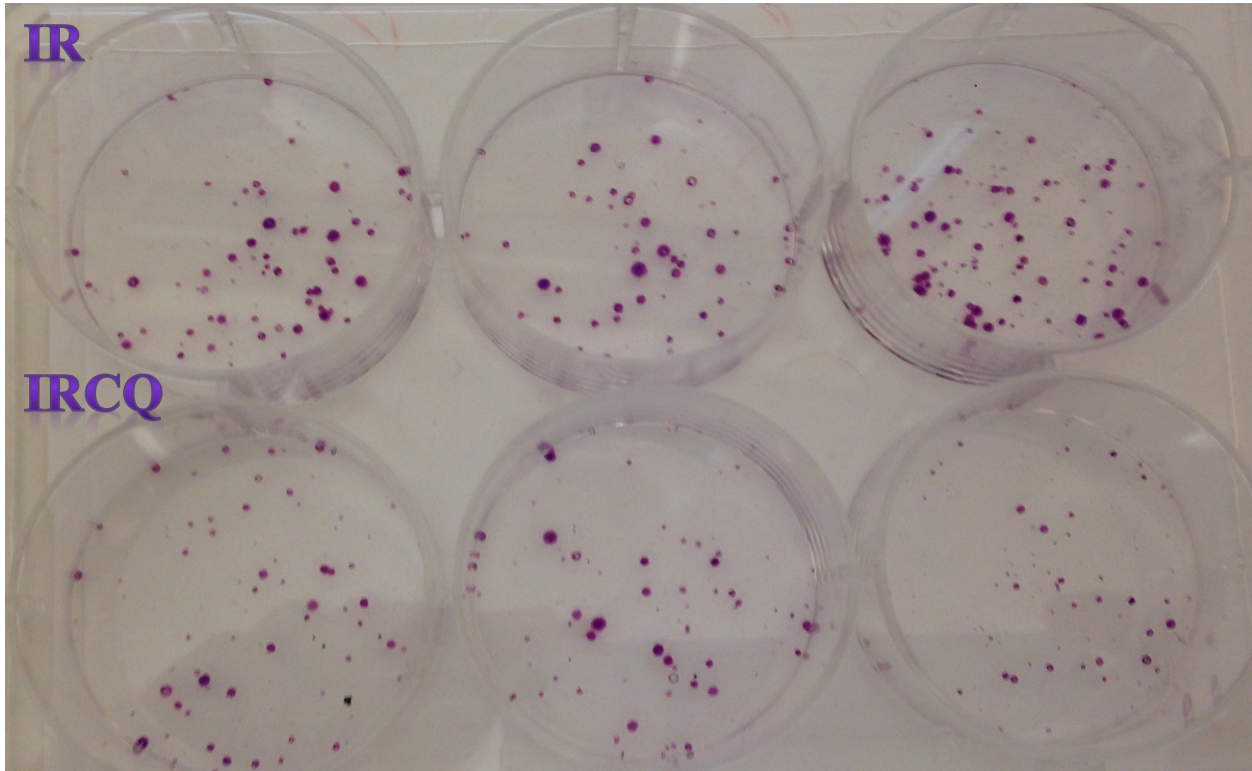


Figure 3.11: Clonogenic survival assay for HN6 cells

This figure shows HN6 cells exposed to IR and IR+CQ 2 weeks after treatment. Cells were plated in 6 well plates each in triplicate .IR: 4 Gy , CQ:5 μ M . After 2 weeks cells were fixed and stained with crystal violet.

This experiment was repeated 3 different times

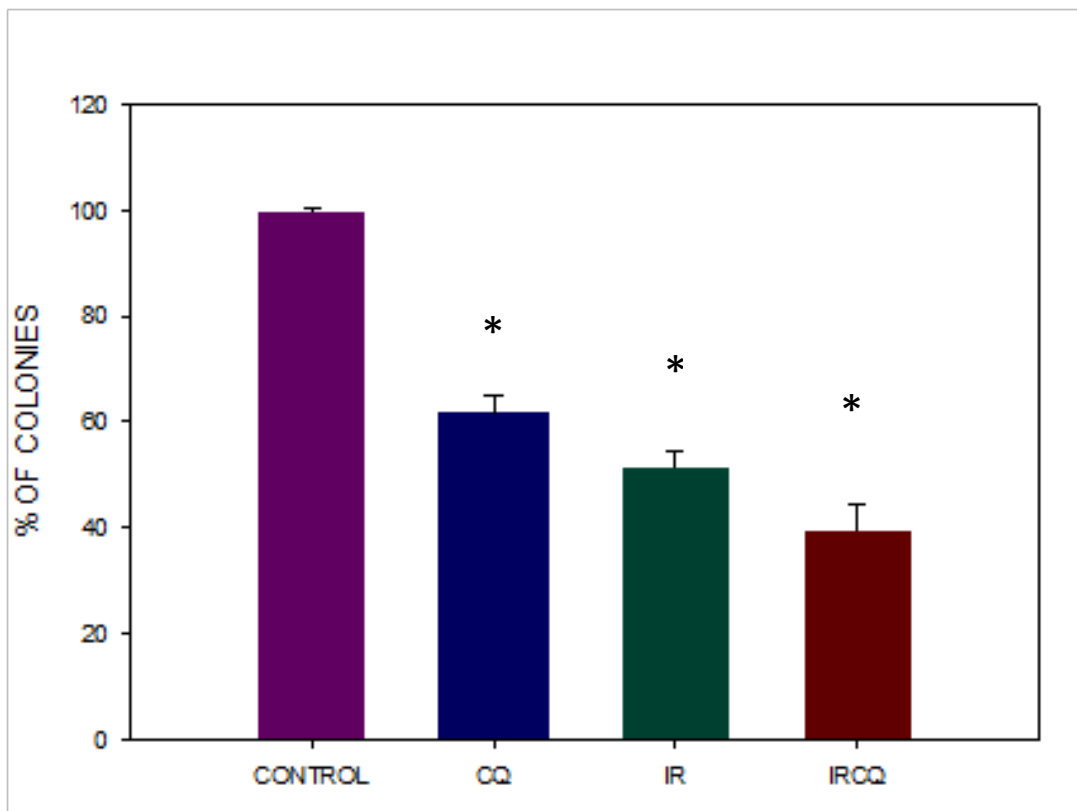


Figure 3.12: Quantification of Clonogenic survival assay for HN6 cells:

. IR and CQ alone have similar effect on the number of colonies also IR+CQ has no significant difference. experiment repeated at least 3 times. IR Dose= 4 Gy ,CQ:5 μ M (* p <0.05 compared to control)

3.5 To confirm that chloroquine is effectively inhibiting autophagy

Acridine orange staining was performed for both cell lines. Acridine Orange is a lysomotropic dye which is trapped in the acidic pH of organelles inside the cells. So when cells induce autophagy the acidic organelles increase and this stain is used widely to identify cells undergoing autophagy. Under microscope a green fluorescent molecule is seen with this stain when it is present in a physiological pH but when it is trapped in an acidic environment it becomes protonated, and emits bright red fluorescence (Klionsky et al, 2007).. Figure 3.14 indicates that there was an increase in orange vesicles after using chloroquine. This occurs because chloroquine inhibits the last step of autophagy as mentioned earlier, and as a result the autophagosomes accumulate in the cells (Fig 3.13 -3.14). These results were confirmed by measuring autophagy with flow cytometry to quantify the acidic vesicles (AVO) and accumulated in the cells (figure 3.13-3.16) HN30 cells shows accumulation of AVO particularly for the combined treatment of IR+CQ, increasing gradually with a significant increase on day 5 (Figure 3.16) and then decrease on day 7. However, HN6 cells show Fig (3.16-3.17) that there increase in the first three days in both IR and IRCQ of AVO formation and followed by low AVO formation in both treatment condition indicating that blocking autophagy did not change the response of cells to radiation induced autophagy. The reason for irradiated HN30 cells not to accumulate AVO is suggestive of flux (completion of autophagy in the HN30 cells treated with IR). In contrast, in HN6 cells, AVO is increased in the first 3 days with a similar increase for both IR and IR+CQ treated cells. This could be indicative of autophagy incompleteness in this cell line and in IR and IR+CQ

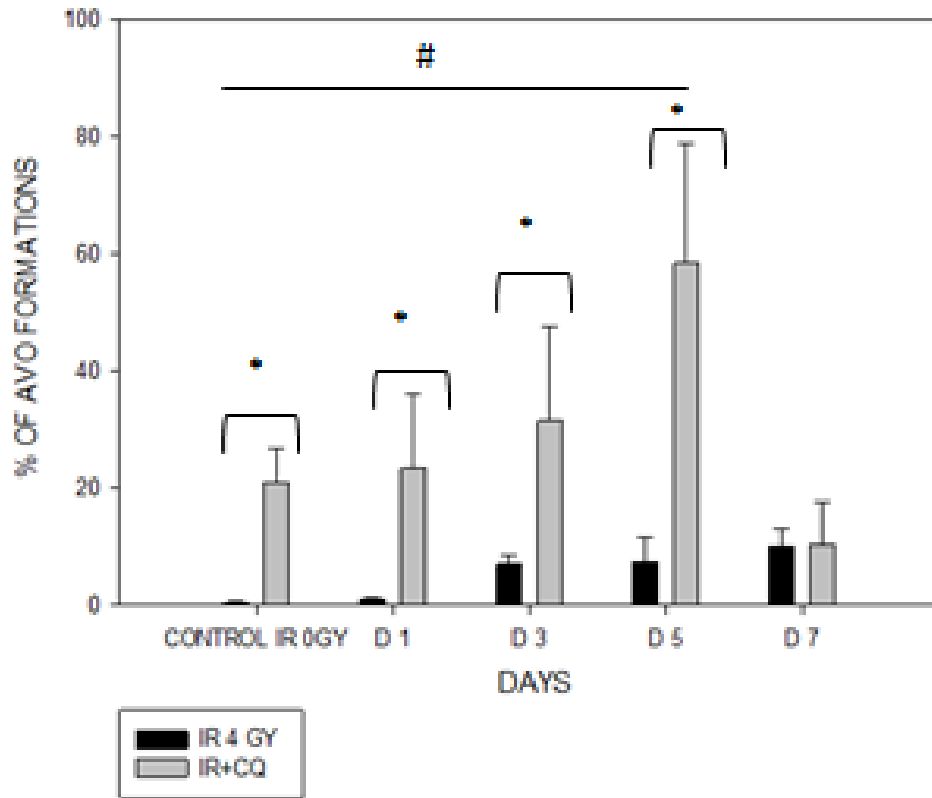
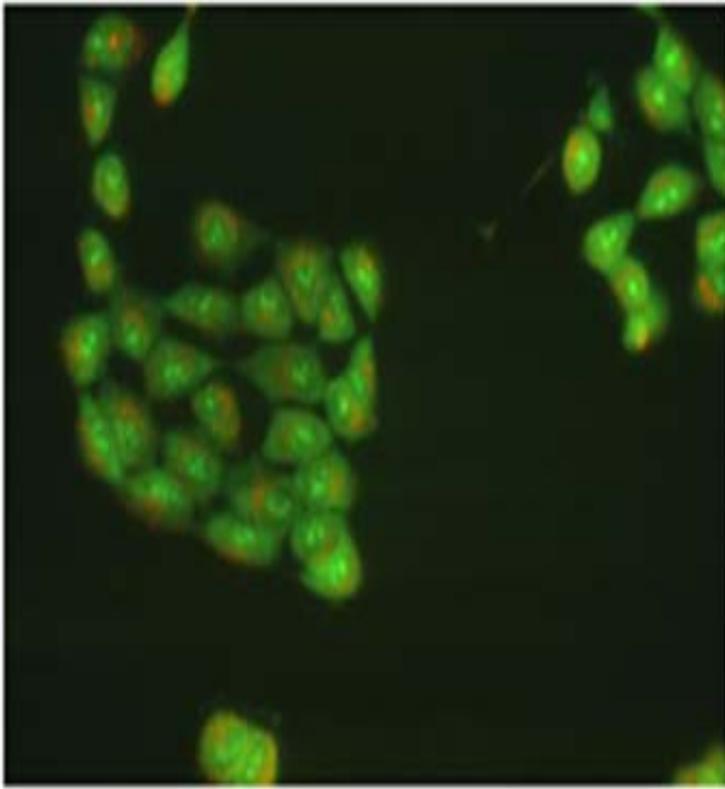


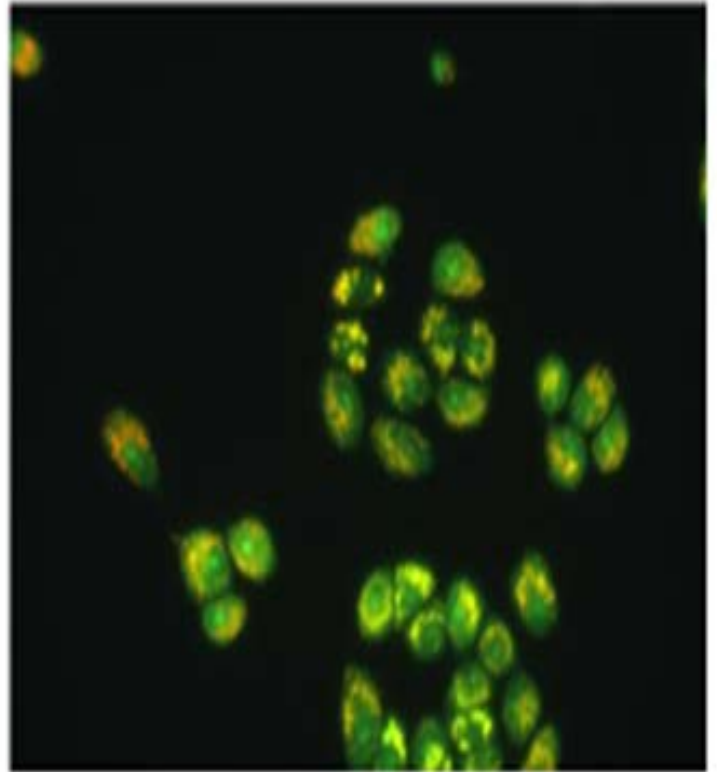
Figure 3.13: Quantification of intensity of autophagy by flow cytometry for HN30 cells

Cells were plated, harvested ,and stained with acridine orange (1:10000). The extent of autophagy was determined based on the number of cell population in quadrants Q2 and Q4 from our raw data. This experiment is the average of three experiment (# p<0.05 compared to control ,

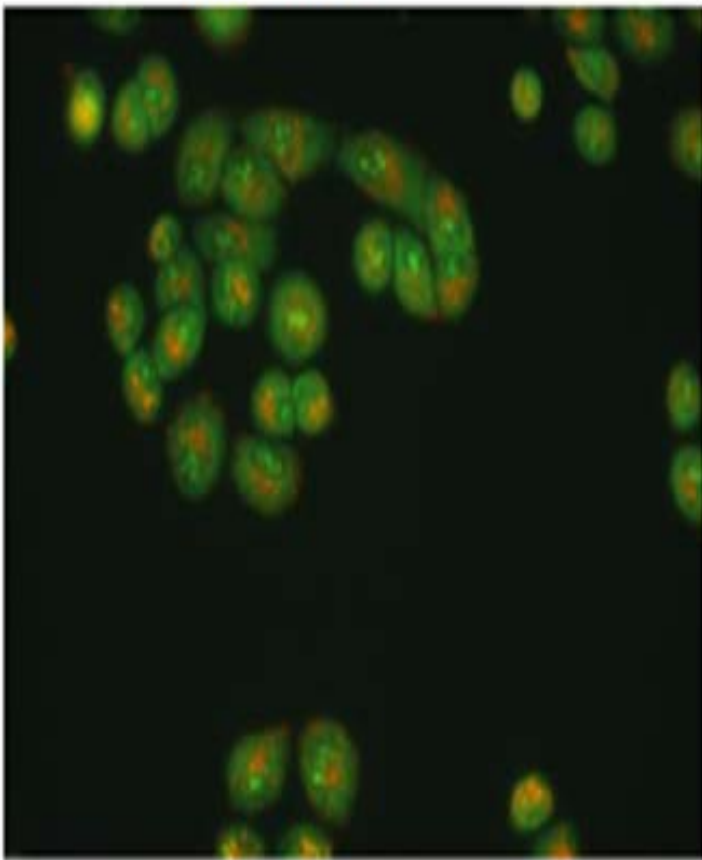
* p<0.05 compared to radiated)



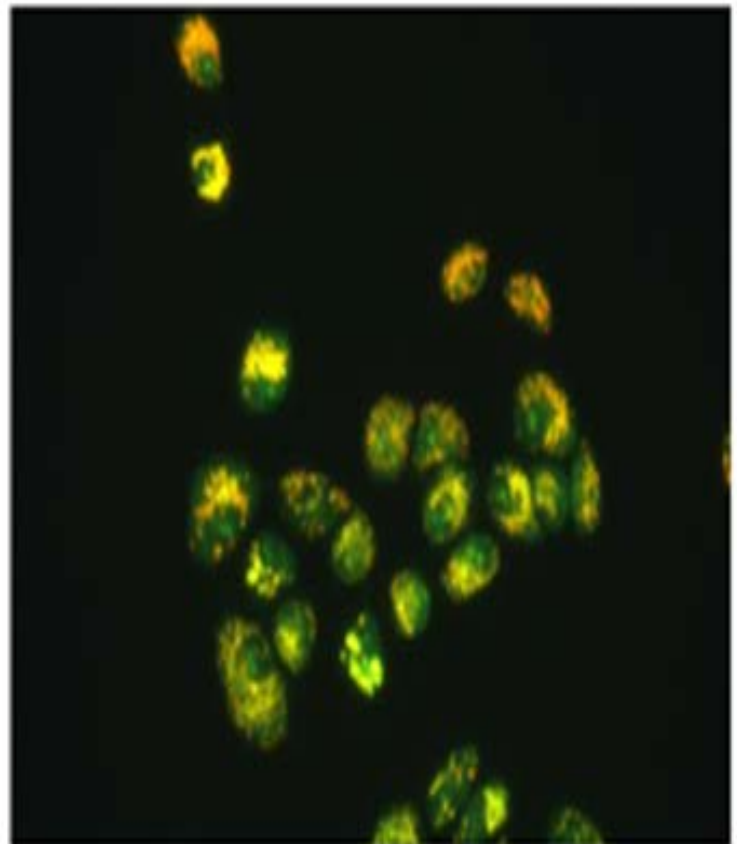
Control HN30



CQ 5 μ M



IR 4GY



IR 4GY +CQ 5 μ M

Figure 3.14: Evaluation of autophagy induction by acridine orange staining in HN30

Cells were plated and treated with IR, 4Gy . At indicated points, acridine orange was added to cells. Images were taken under a fluorescent microscope at a magnification power of 20X. Three different experiments were performed to confirm the result.

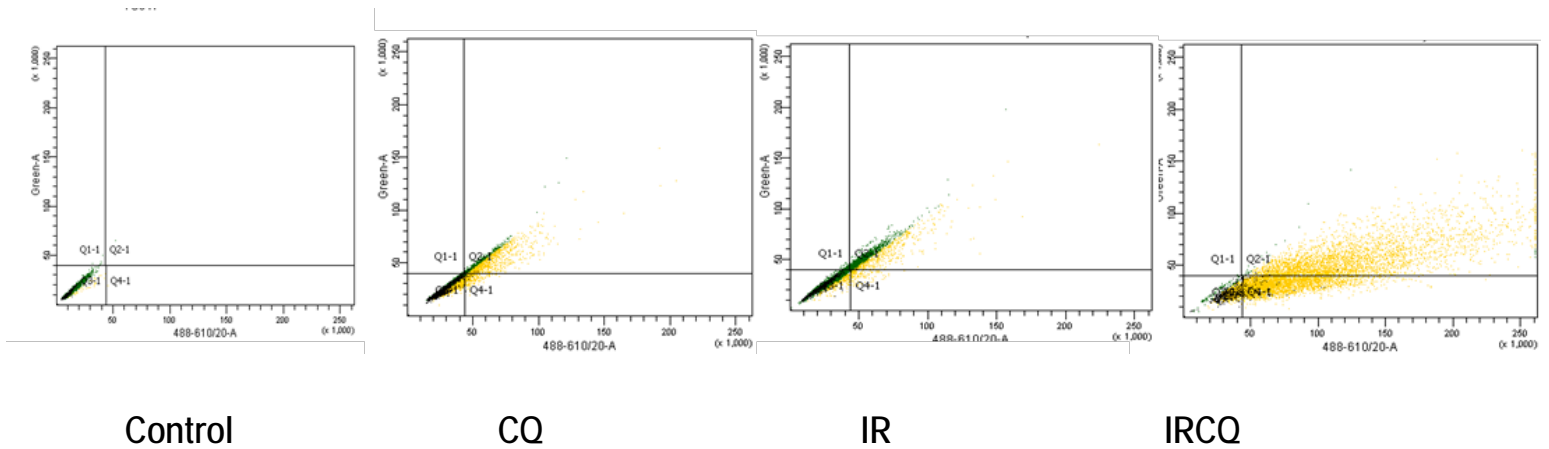


Figure 3.15: Flow cytometry to assess autophagy in HN30 cells

Data show extent of acidic vacuole formation in HN30 cells day 5. Autophagy was counted based on the number of cell population in quadrants Q2 and Q4 (green color indicate the green fluorescence and no AVO and the yellow color indicate AVO which emits orange color)

X axis represent green fluorescence and Y axis represent yellow fluorescence

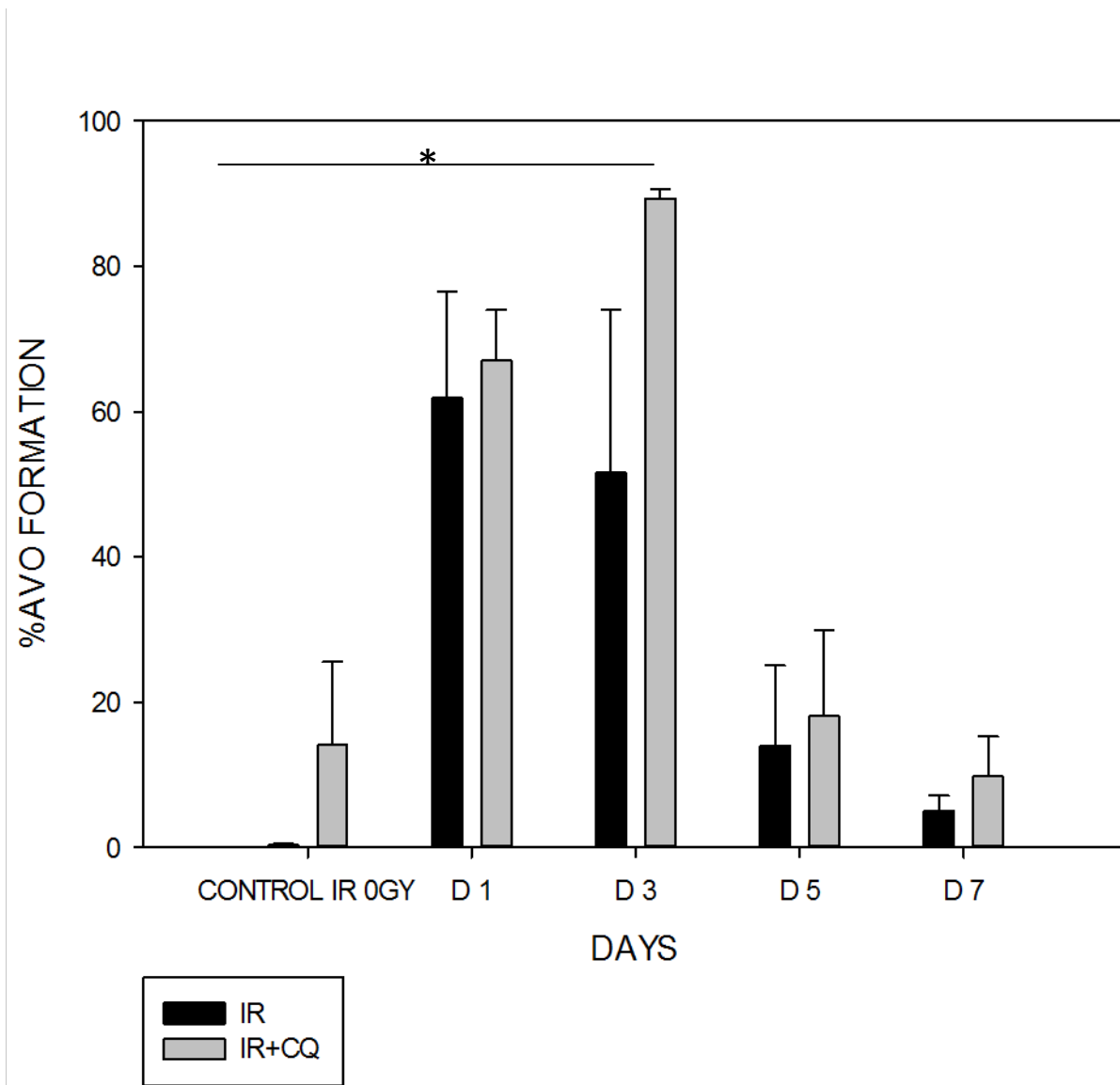
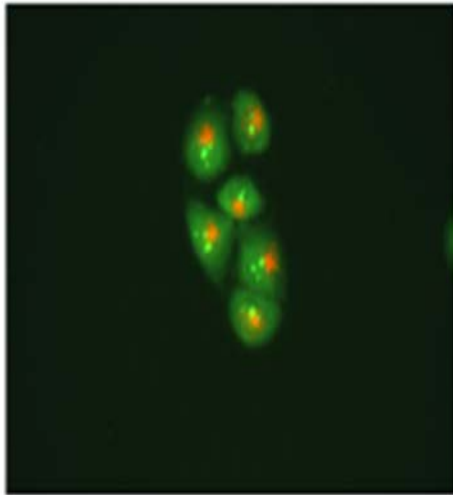
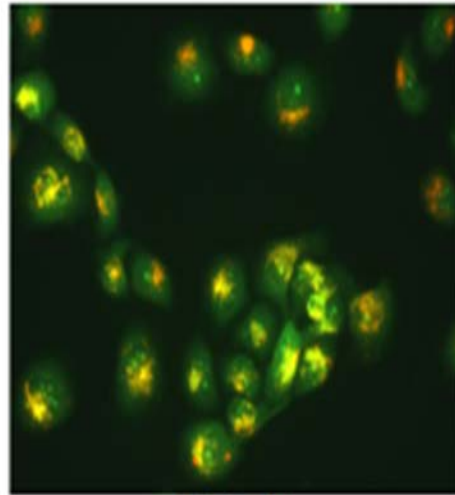


Figure 3.16: Quantification of intensity of autophagy by flow cytometry for HN6 cells

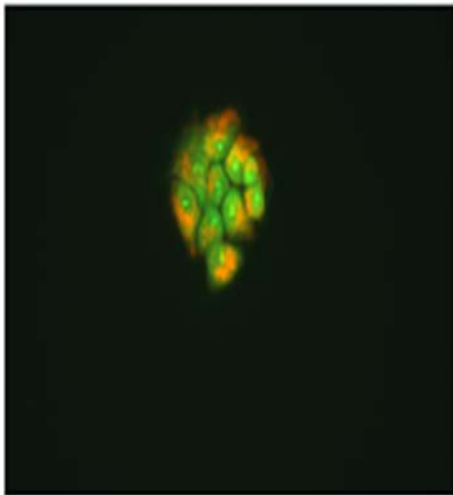
Cells were plated, harvested, and stained with acridine orange .The extent of autophagy was counted based on the number of cell population in quadrants Q2 and Q4 from our raw data. This experiment is the average of three experiment (* p<0.05 compared to control)



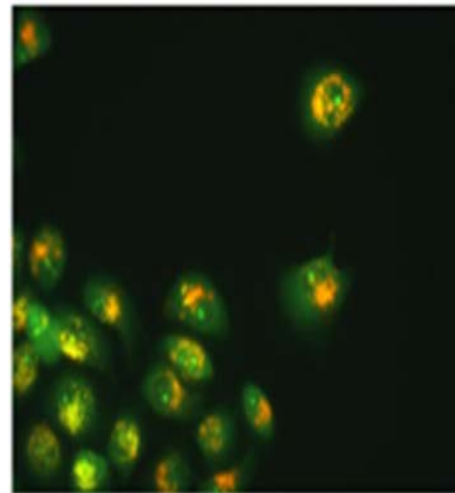
Control HN6



CQ 5 uM



IR 4Gy



IRCQ

Figure 3.17 Evaluation of autophagy induction by acridine orange staining in HN6 cells

Cells were plated and treated with IR, 4Gy. Acridine orange was added to the cells. Images were taken under a fluorescent microscope at a magnification power of 20X. Three different experiments were performed to confirm the results.

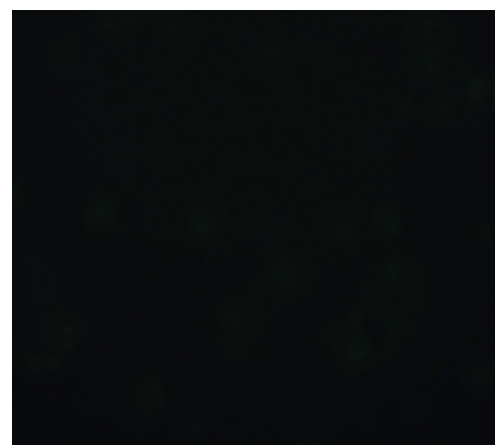
3.6 Radiation induces minimal apoptosis in HN30 and HN6 cells

DAPI and TUNEL staining are commonly used to look for apoptotic cells. DAPI (4, 6-Diamidino-2-phenylindole) staining will show a rounded nucleus with a clear margin in normal cells while the margin will not be nice and clear in apoptotic cell's nucleus (Tschopp et al, 1993). That is because the apoptotic cells have a characteristic fragmented DNA (Tschopp et al, 1993). In the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay, fluorescent nucleotides are attached to the 3'-OH end of fragmented DNA, which emits bright green fluorescence in apoptotic cells.

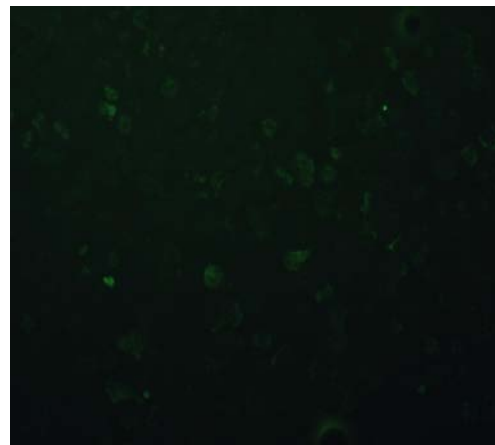
In cytoprotective autophagy, inhibition of autophagy will lead to sensitization and cellular death and it could die through apoptosis. (Jin H et al, 2011, Bristol et al., 2012). Another important part was to check if radiation alone induces any apoptosis in HN30 and HN6 cells in order to study the mode of cell death.

Figures (3.18-3.19) HN30 cells undergo minimal apoptosis upon radiation. Taxol (1 μ m for 24 hrs.) was used as a positive control for apoptosis. In Figure 3.20, irradiated HN30 cells reveal lack of increased fluorescence in the flow cytometer for annexin V/PI staining indicating lack of induction of extensive apoptosis.

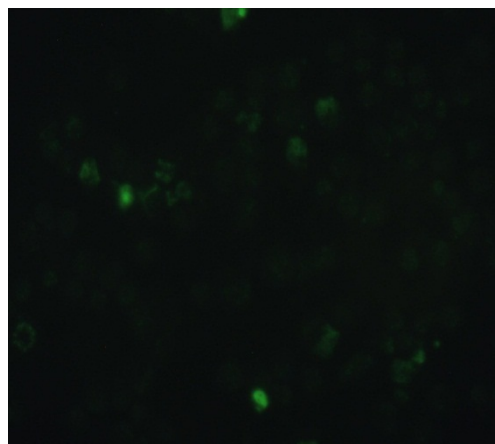
HN6 cells has also transient apoptosis increased with the increase of AVO formation in both IR and IR+CQ, and that complies with the time course when cell death was seen in the first few days. However, the overall is minimal apoptosis seen in FACs for HN6 (figure 3.21)



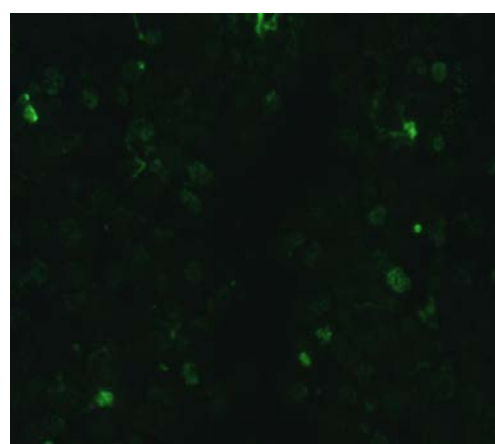
Control



CQ 5 uM



IR 4 Gy



IR 4Gy +CQ 5 uM

Figure 3.18: TUNEL assay for HN30 cells:

HN30 cells were plated in 6-well plates each condition in triplicate and treated with the indicated concentrations of CQ with and without radiation. At Day 4, Images were taken under a fluorescent microscope with magnification power of 20X., N=2

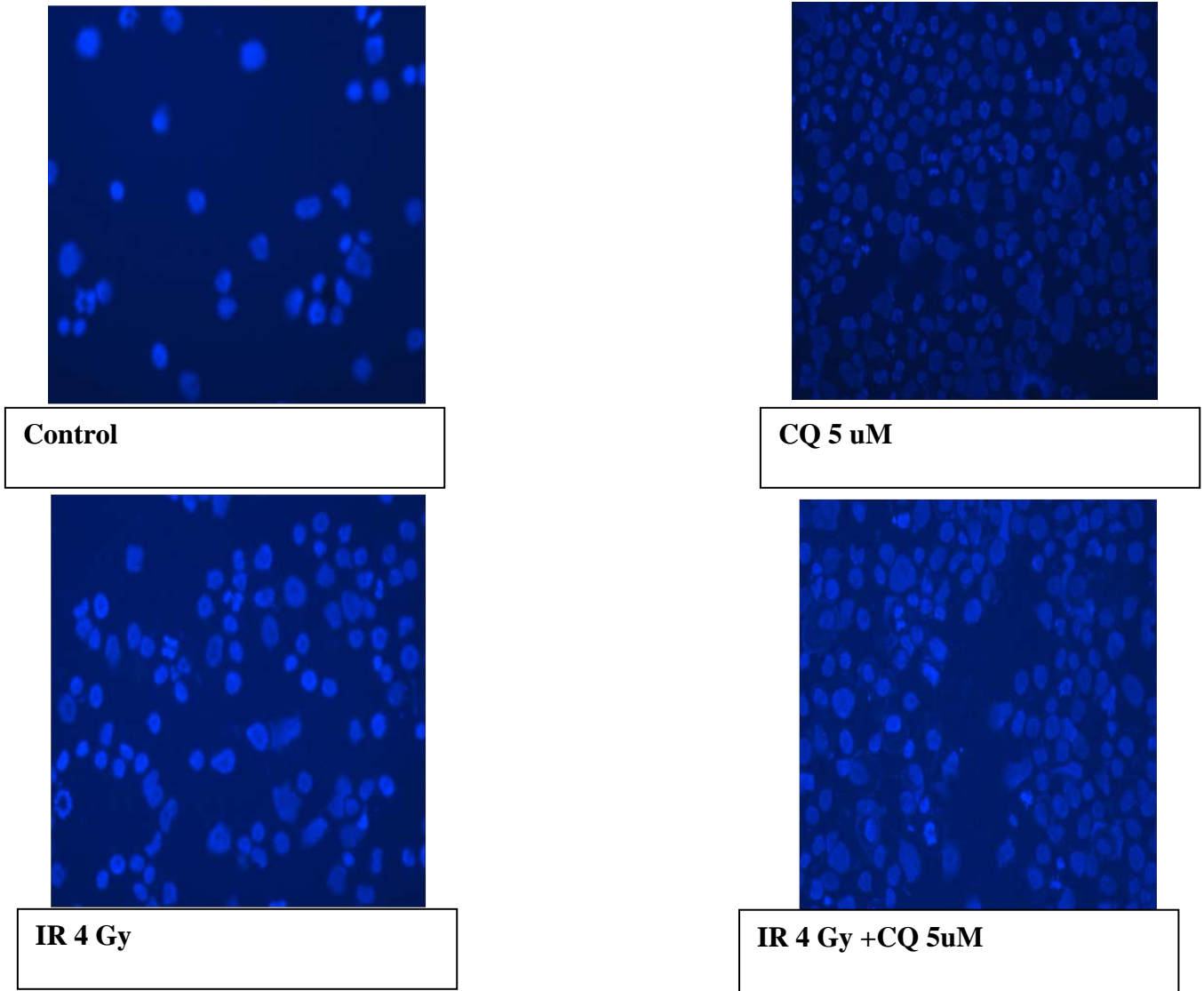


Figure 3.19 DAPI staining for HN30

HN30 cells were plated in 6-well plates and treated with the indicated concentration. At Day 4, drug was removed and a dilution (1:10,000) of DAPI in PBS was added to stain the nuclei. Images were taken under a fluorescent microscope with magnification power of 20X. This experiment was done twice ,

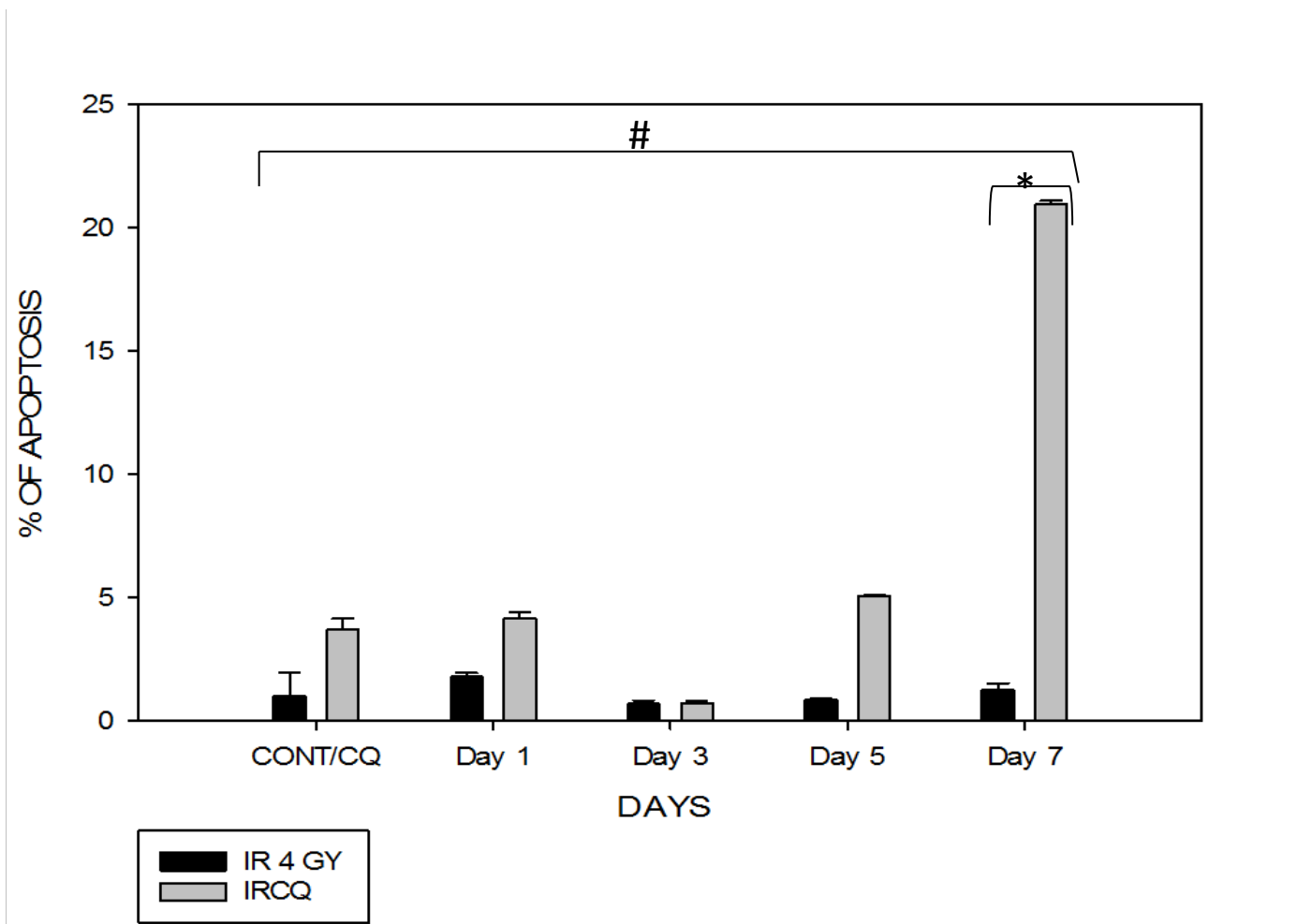


Figure 3.20: Flow cytometry to check for apoptosis in HN30

Cells were treated with IR 4Gy±CQ: 5µM for one, three, five and seven days and stained with Annexin V and PI to assess whether cells die through apoptosis and/or necrosis. The raw data show four quadrants; Q1 resembles the necrotic cells only, Q2 resembles the late apoptotic cell population, Q3 resembles cells that are neither apoptotic nor necrotic, and Q4 indicates early apoptosis. The percentage of apoptotic cells is counted based on percentage of cells in both Q2 and Q4. This experiment was performed twice and each experiment was in triplicate (* p < 0.05 compared to radiation alone, # p<0.05 compared to control)

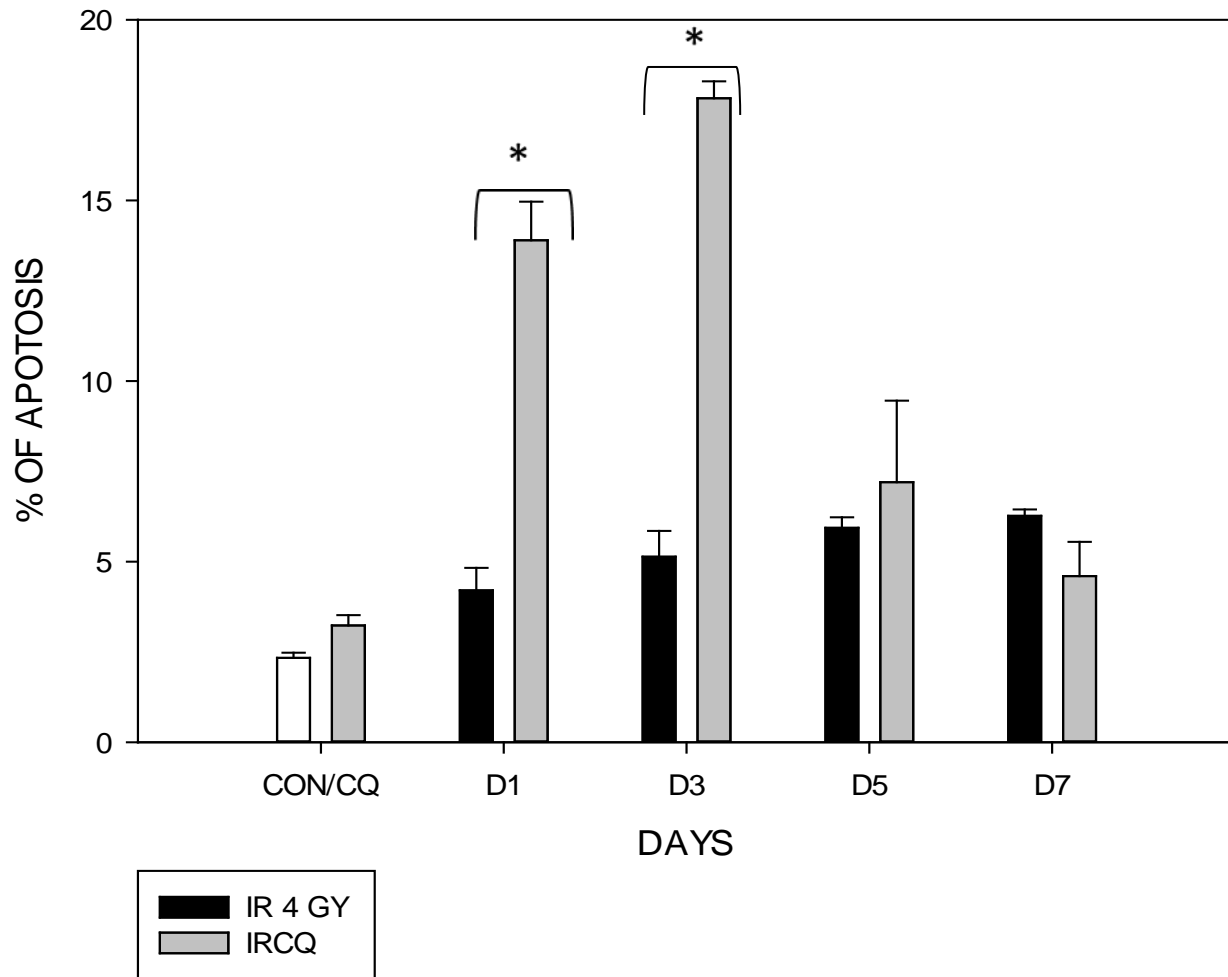
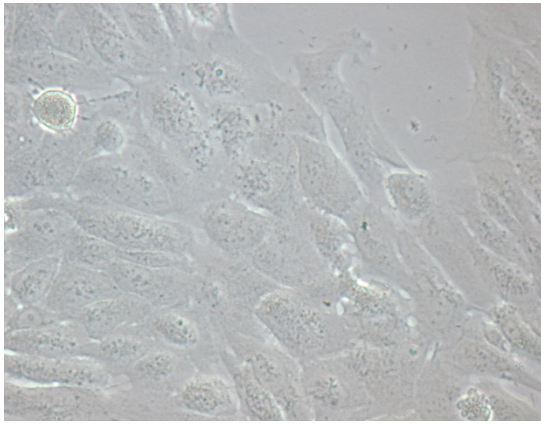


Figure 3.21 Flow cytometry to check for apoptosis in HN6

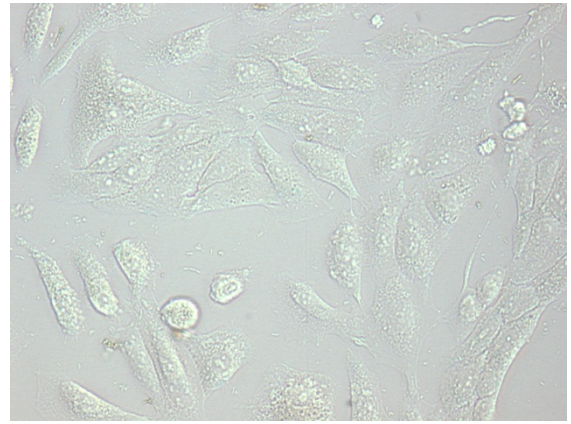
Cells were treated with IR 4Gy±CQ: 5µM for one, three, five and seven days and stained with Annexin V and PI to assess whether cells die through apoptosis and/or necrosis. The raw data show four quadrants; Q1 resembles the necrotic cells only, Q2 resembles the late apoptotic cell population, Q3 resembles cells that neither apoptotic nor necrotic cells, and Q4 indicates early apoptosis. The percentage of apoptotic cells is counted based on percentage of cells in both Q2 and Q4. This experiment was performed twice.

3.7 Checking for senescence in HN30 cells

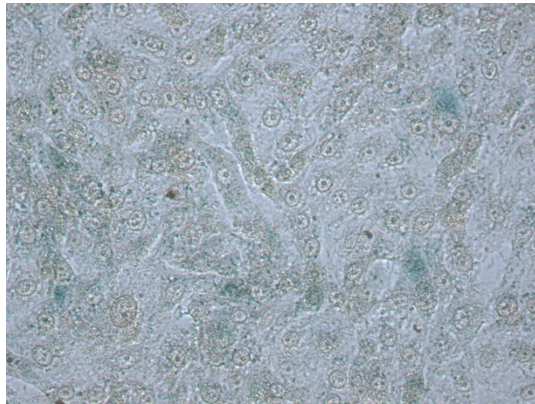
Senescence is a state of cellular growth arrest. (Serrano M. et al., 2006) .HN30 were treated with CQ± IR and then checked for senescence. In Figure 3.22 IR+CQ showed reduced beta galactosidase staining compared to radiation alone. This could mean there is a link between irradiation induced autophagy and senescence; that is because senescence was reduced when autophagy was inhibited.



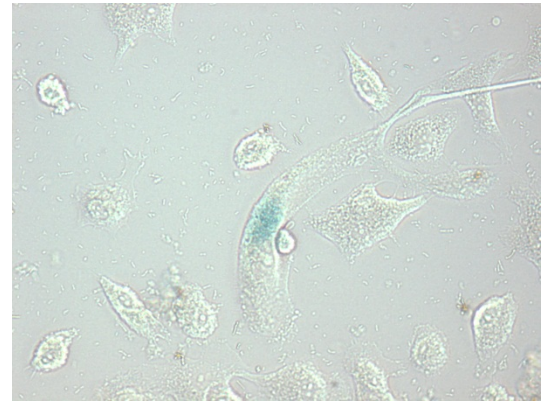
Control



CQ



IR



IR + CQ

Figure 3.22: Effect of autophagy inhibition by CQ on senescence in HN30 cells

Cells were fixed and incubated in a CO₂ free incubator with staining solution for 24 hours to maintain pH of 6 .All images were taken under white light inverted microscope. Radiation dose= 4Gy. 20X,

Discussion:

Radiation therapy is one of the primary treatments for patients with head and neck cancer (Masuda & Kamiya, 2011). A drug that could be used for making head and neck cancer more radio sensitive could be used to reduce the long treatment periods, the chance of recurrence, and the possible side effects of radiation. When cells are irradiated they undergo autophagy (Rodemann et al, 2011). Autophagy is a basic catabolic mechanism in which cells break down organelles as a source of fuel and metabolic precursors (Przyklenk et al, 2010). Autophagy can be cytoprotective, helping the cell to survive during stress such as nutrient deprivation (or radiation) or it can be cytotoxic, leading the cell toward death (White E et al, 2010).

When HN30 and HN6 cells were irradiated with 4Gy they showed growth arrest followed by recovery with a slight degree of cellular death in the HN6 cells. Also acridine orange staining was used in both cell lines (HN30 & HN6) to indicate that the irradiated cells showed an increase in the cellular acidic compartment volume which could be used as an indicator of autophagy and therefore staining of the acidified autophagosome is used as an autophagy marker. (Paglin et al., 2001). After that, autophagy was quantified by flow cytometer and also showed an increase in the AVO formation in irradiated cells in both cell lines.

Blocking autophagy was done by using a pharmacological inhibitor drug (chloroquine (CQ)) which blocks the last step of autophagy by interfering with the acidic pH of the lysosome which will lead to inhibition of the lysosomal hydrolases enzymes responsible for the degradation of vesicles and accumulation of undigested vesicles (autophagosomes) (Rodemann et al, 2010, Zisterer et al, 2012). Earlier studies in our laboratory data showed that MCF-7 and ZR-5 breast tumors when treated with an autophagy inhibitor such as CQ were sensitized to radiation (Bristol

et al, 2012 ,Wilson et al, 2012) . Sensitization is a method used to make a two drugs used together more effective than each one alone (P.269-270,2nd edition, advances in radiation oncology in Lung cancer, Brady L.W., Heilmann H.P., Molls.M. Nieder.C.). The effects of autophagy inhibition by CQ were observed by assessment of sensitivity (time course for cell viability and clonogenic survival assays). HN30 cells (p53 Wild type) (Patel Vet al, 2000) were radiosensitized upon treatment with CQ 4 hours prior to radiation while HN6 cells (p53 mutant) (Patel Vet al, 2000) did not show radiosensitization. This may be because HN30 is having cytoprotective autophagy and when this protective mechanism the cells use to survive stresses is blocked with chloroquine treatment those cells tend to be more sensitive to radiation and die. While HN6 is having non protective autophagy and thus blocking autophagy did not affect the growth pattern and radiation sensitivity.

In HN30 cells, acidic vacuoles organelle formation (AVO) was present in irradiated cells but increased markedly with the addition of CQ to the irradiated cells .This could be expected because CQ inhibits the last step and lead to accumulation of autophagosomes . The reason for IR treated cells not to accumulate a very high number of AVO as with IR+CQ would be suggestive of flux (completion of autophagy in the HN30 cells treated with IR alone). So there is AVO formed and accumulated in IR but not to the extent of the IR+CQ had. And it is thought that autophagy is going fast into completion in IR cells and it can be detect when it stopped from being completed with CQ.

In HN6 cells, AVO is increased in the first 3 days with a similar increase in both IR and IR+CQ treated cells this could be indicative of autophagy incomplection in this cell line and in both IR

and IR+CQ conditions. And that it is why when autophagy is blocked it does not show a significant difference between IR and IR+CQ.

Those previous findings should that HN30 cells became sensitized to radiation upon blocking autophagy by using CQ .This could mean that those cells when exposed to radiation they induce a protective mechanism (cytoprotective autophagy) and when it is blocked cells die. On the other hand, HN6 cells did not have either protective autophagy or cytotoxic autophagy; we have termed this non protective autophagy because blocking it by CQ did not show sensitization or increased cellular growth. It only showed similar or less than additive effect of both IR and CQ. In order for autophagy to be cytotoxic and pro-death, then inhibition of autophagy protects the cells from the radiation treatment (Gewirtz et al, 2009).

Assessment of apoptosis by flow cytometer studies using Annexin V/PI staining in HN30 and HN6 showed an increase in apoptosis after a huge accumulation of AVO in both cell lines when autophagy induced by radiation was inhibited; however, the absolute extent of apoptosis was relatively low and/or transient Also DAPI/TUNEL staining was done on HN30 cells treated with the IR and IR+CQ. Again autophagy inhibition by CQ did not show significant increase in cell death by apoptosis which means that apoptosis is not the main mode of radiosensitization in HN30 cells . HN6 cells showed transient apoptosis in the first few days which is consistent with the growth death seen in the first few days in the time course studies when cells are irradiated. Moreover apoptosis did not increase with autophagy inhibition. HN6 cells also showed lack of radiosensitization with simply additive effects using autophagy inhibitors. Cellular death due to autophagy without apoptosis is still not clear. Debate continues if autophagy itself acts as a mode of cell death or just leads to some other form of cell death. (Codogno et al, 2011).

Senescence was also assessed in HN30 cells. Data showed growth arrest in the cellular viability studies which could mean induction of senescence. β galactosidase staining showed some increase in the blue dye which is a marker of senescence in IR treated cells and a minimal senescence induction increase with IR+CQ. This could mean that autophagy and senescence are related cause when autophagy is inhibited senescence was reduced. Some studies showed that autophagy might be contributing to oncogene induced senescence (Young et al ,2009), and studies in our laboratory demonstrated a close correspondence between autophagy and senescence in response to doxorubicin (Goehe RW et al, 2012).

In vivo studies in animals and clinical trials are in progress to address if autophagy inhibition by using chloroquine or hydroxychloroquine can chemo-sensitize or radio-sensitize tumors (Kelekar et al, 2007, Amaravadi et al, 2007).

In vivo studies in animals and clinical trials are in progress to address if autophagy inhibition by using chloroquine or hydroxychloroquine can chemo-sensitize or radio-sensitize tumors (Kelekar et al, 2007, Amaravadi et al, 2007).

One of the major differences between the two cell lines (HN30 & HN6) is the p53 status. HN30 has a wild type p53 and HN6 has a mutant p53 (Patel V and Yeudall WA, 2000) .In our laboratories, MCF-7 are p53 wild type cells that showed cytoprotective autophagy and upon inhibition using either pharmacological or genetic inhibition of autophagy, radio-sensitization was observed (similar to the outcome in the p53 wild type HN30 cells). In contrast, Hs578t

breast tumor cells are p53 mutant and failed to demonstrate radio-sensitization upon inhibition of autophagy just like the HN6 cells. So p53 could be one of the causes of different responses

Conclusions: Autophagy induced by radiation in HN30 cells appears to be cytoprotective and when inhibited by CQ the cells lose an important defense mechanism and die. Autophagy induced by radiation in HN6 cells is non-protective, which may be a consequence of the fact that autophagy is not going to completion in this cell line

Future studies:

In order to check for completion of autophagy, p62 Western blot could be used. P62 ubiquitin protein recognizes toxic cellular waste, which is then undergo autophagy (Tor E and Harald S, 2010) P62 Western could show the degradation of p62 in irradiated HN30 cells and accumulation with IR +CQ treatment, which would support our hypothesis for cytoprotective autophagy is due to the completion of autophagy in the IR treated cells and the incompleteness of autophagy in IR+CQ treated cells. Also we could check for LC3 I conversion to LC3 II by either using Western or RFP-LC3 (Red fluorescent protein). LC3 is one of the proteins that form the autophagosome and upon activation of autophagy, LC3 I is changed to LC3 II by the aid of ATG7 (Tanida, I D et al, 2008) and monitoring the LC3 I conversion to LC3 II by Western blots will confirm autophagy or with the red fluorescent protein – LC3 where cells get transfected with RFP-LC3 after that the diffuse and punctate staining will be monitored and counted using fluorescence microscopy.

Moreover to study the outcome of autophagy inhibition on radiation sensitivity in HN30 and HN6 cells, additional pharmacological inhibitors for autophagy should be used to confirm the result. This could include 3MA which inhibits class I and class III phosphoinositide 3-kinase (Liu J et al, 2012). With the consideration that 3MA can be nonspecific because it can act on class I PI3K which inhibit mTOR and prompt autophagy instead of inhibiting autophagy (Shen et al, 2011).

Using genetic methods should be performed. By silencing autophagy gene atg5 or atg7 using siRNA techniques, which are genes essential for autophagy, should cause autophagy inhibition and confirm what was seen in both cell lines. Another genetic method is to silence p53 in HN30

cells or express p53 in the HN6 cells. Overexpression will be done by first knock down the mutant p53 then over express a functional p53 in the HN6 cells .This method could confirm if the autophagy in order to be protective it need a functional p53. Such inhibition would provide an indication as to whether p53 plays a key role and then cyto protective autophagy in this case will be altered.

To study the autophagy and senescence relationship; time course studies for p62 degradation besides p21 induction. . p21 is a very essential protein in senescence pathway and it is increased during senescence (Campisi., 2008). And it has been shown in the data that when autophagy was blocked by CQ, senescence was decreased so checking the relation between p62 and p21 using western could help to understand the relationship between autophagy and senescence.

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