Junior practical training

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Radiation-induced molecular modulations in thyroid cells cultured under iodine-deficiency

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Content

Abstract

We are exposed to natural radiations from different sources on a daily basis. In addition to the background radiation, some people receive artificial radiations for diagnostic or therapeutic purposes. It is already established that high doses of radiation induce harmful effects on the cells but the effects of low doses of radiations are still unclear. Besides radiation, there are worldwide two billion people suffering from iodine deficiency and the latter is known to have many consequences such as endemic goiter, growth retardation and it is a risk factor for thyroid cancer.

As both external radiation and iodine deficiency induce harmful effects on thyroid cells separately and that there are little knowledge available about their combined effects, the current study was undertaken to understand the basic mechanisms of thyroid cancer risks after low dose exposure to external radiations under iodine deficiency. One potential mechanism by which external radiation and iodine deficiency would merge their harmful effects is the increase of ROS production at a level that becomes deleterious enough to trigger tumor initiation. This study shows that irradiation and iodine deficiency induce molecular changes in the thyroid cells that lead to the activation of pro-survival pathways and apoptosis escape, two mechanisms characteristic of cancer cells. Thus irradiation and iodine deficiency may be considered as risk factors for thyroid cancer initiation. These findings are important for radioprotection regulation, especially for those patients receiving high doses of radiation for therapeutic reasons as nowadays the iodine status of the patients is not taken into account before the start of the treatment.

Introduction

1. General introduction

we are exposed to radiations from different sources on a daily basis. Natural radiations come mainly from two sources: earth or outer space. Radiations that come from the outer space are called cosmic radiation or cosmic rays, while the radiations from the earth come from radioactive substances in the earth crust such as soil, air and from sunlight which are mainly infrared and ultraviolet radiations. Natural sources of radiations represent the major contributor to our daily exposure and radon gas is the major contributor to these natural radiations. Radon gas, which can be found in many places such as in the southern area of Belgium, is commonly found in rocks and soil where it can escape from them to enter the water and the air. The exposure to radiations may be form artificial sources as well such as exposure for industrial or medical diagnostic/therapeutic purposes. These medical applications become widely used due technological progress in these fields. Example of these applications are X-ray, Computer Tomography Imaging (CT-scan) and Positron Emission Tomography (PET-scan).

The effects of high doses of radiations are known. The shape of the dose-response curve for high doses is linear as increasing the radiations doses will increase the harmful effects on human cells. High doses of radiations are associated with increased thyroid cancer, this have been seen after the Chernobyl accident in 1986. The risk of thyroid cancer was dramatically increased in children that were exposed to radiations[1-8]. Unlike high doses, the effects of low doses of radiations are still not fully understood.

Within the general population which is subjected to low doses of radiations, there are billions of people suffering from iodine deficiency. Indeed, iodine deficiency is a significant health problem in developed and developing countries[9], according to world health organization, two billion people are suffering from iodine deficiency worldwide, this lead to many consequences such as endemic goiter, growth retardation, intellectual

impairment, and increased pregnancy loss and infant mortality[9, 10]. In addition to that, deficient iodine intake may be associated with increased thyroid cancer incidence[10-14].

There is little knowledge about the combined effect of iodine deficiency and low doses of external radiation on the incidence of thyroid cancer. After the Chernobyl nuclear work station accident, epidemiological studies showed that the high incidence of thyroid cancer is associated with the high radiation dose together with iodine deficiency which was measured in the urine of the affected individuals in the contaminated areas around the accident [2, 3, 7, 15].Since the effects of low doses of external radiations are unclear and that iodine deficiency is a worldwide health problem that was associated to thyroid cancer, this research has been performed to study the combined effects of low doses of external radiations and iodine deficiency on thyroid cells.

2. Objectives

The overall objective of this work is the study of the combined effect of low doses of external radiations(X-rays) and iodine deficiency on thyroid cells in *vitro* and to investigate the pathways triggered by this combined effect. We hypothesize that low doses of radiations and iodine deficiency will increase Reactive Oxygen Species (ROS) production within the cells and that would lead to deleterious effects on the cells.

In brief, normal thyroid cell line (FRTL-5) from rat origin were subjected to a co-treatment with low doses of radiation and iodine deficiency.

The specific objective of this work is the investigation of the mechanisms which are involved in precancerous transformation such as cell survival (Phosphatidylinositide 3 Kinase/AKT (PI3K/AKT),Nuclear Factor Kappa-B (NF-kB)), proliferation (Mitogen Activated Protein Kinase/Extracellular signal Regulated Kinase (MAPK/ERK), and apoptosis escape.

3. Introduction to radiation

Radiations are the emission of energy as electromagnetic waves or as moving subatomic particles. Electromagnetic radiation are classified into two main categories: ionizing radiations or non-ionizing radiations. Ionizing radiations have enough energy (>13,6 eV) to ionize the matter by liberating an electron from atoms or molecules, this includes alpha and beta particles, neutrons, X-rays andγ-rays. Non-ionizing radiations are relatively low energy radiations(<13,6 eV)that can't ionize the matter and they are considered to be less dangerous(*ie*, microwaves, radiowaves, infrared, UV and visible light).The basic unit to measure exposure to ionizing radiations is a Sievert (Sv),this unit measures the biological effect of absorbed radiations (referred to as an "effective dose"). Radiations can also be measured by Gray unit (Gy), that measures the absorbed dose and it is defined as energy per mass(1Gy= 1Joule/kilogram).Ionizing radiations may have serious effects on the body. They can cause skin lesions, cancer, heart disease, and in pregnant women can harm the fetus, induce miscarriage, cause congenital malformations or mental retardation depending on the gestational stage at the moment of irradiation. The harmful effects of radiations are linked to the transfer of energy to atoms and molecules in the cellular structure. Ionizing radiation causes atoms and molecules to become ionized or excited. These excitations and ionizations can induce DNA damage resulting in single strand break or double strand breaks, protein/DNA cross linking, reduce DNA repairing capacity, alter DNA methylation, which lead to cell damage or mutations[16-18]. Radiation can also produce Reactive Oxygen Species (ROS) which cause significant damage to the DNA and to other cell structures and activates many precancerous pathways[16, 19].At the molecular level, there are several pathways induced by radiations. These pathways include among others, PI3K/AKT pathway, MAPKpathways,p53 pathway, in addition to effect on different cytokines and molecules signaling such as NF-KB, IL (interleukin)-1, IL-6, transforming growth factor (TGF)-beta and tumor necrosis factor (TNF)-alpha[20-27].

4. Introduction to thyroid physiology

Thyroid gland, located at the neck below the thyroid cartilage, is important to regulate the growth and to maintain many important functions in the body. This butterfly shaped endocrine gland synthesizes thyroid hormones, triiodothyronine (T3) and thyroxine or tetraiodothyroxin (T4), from iodine.

The hormone production starts with the synthesis of the thyroglobulin (Tg) in the rough endoplasmic reticulum of the thyrocytes then it is secreted in the colloid which is in the lumen of the thyrocytes. The iodide present in the blood stream is actively pumped into the thyrocytes by the Sodium/Iodide Symporter (NIS). Iodide enters the colloid passively via the pendrin transporter which is located at the apical side of the thyrocytes. There the iodide is oxidized to iodine by the Thyroid PerOxidase enzyme (TPO).The iodine and thyroglobulin form conjugate together that enter the thyrocyte cells by endocytosis and inside the cell undergo proteolysis to form T3 and T4 as explained in figure 1. Thyroid hormone synthesis is regulated by Thyroid Stimulating Hormone (TSH) released from the pituitary gland, and TSH level itself is regulated by Thyrotropin Releasing Hormone (TRH) from the hypothalamus.

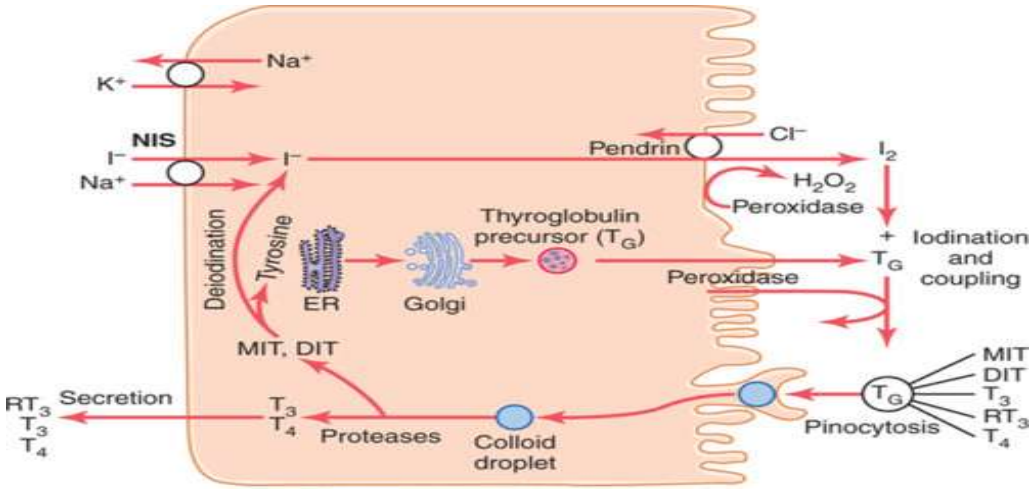


Figure1: synthesis of thyroid hormones*(Guyton and Hall Textbook of Medical Physiology, 12th Edition).*

The main source of iodine is dietary food such as seafood, meat and iodized salt. Iodine deficiency cause reshaping of the microvasculature of the thyroid[28]which ensure a steady delivery of iodine to the thyroid gland. At the molecular level, iodide deficiency induces angiogenic reaction by releasing VEGF-A (Vascular Endothelial Growth Factor) from normal thyrocytes via reactive oxygen species and hypoxia induced elements dependent pathways[29, 30].

Thyroid cancer can be classified into papillary, follicular and medullary thyroid carcinoma. Papillary Thyroid Carcinoma (PTC) is the most common type of thyroid cancer. This cancer forms in follicular cells in the thyroid and grows in small finger-like shapes. It can be induced either naturally or by radiation,. In the latter ,it accounts for 85% of thyroid cancers due to radiation exposure. Iodine deficiency is a risk factor for PTC as well. Chromosomal translocation and point mutation in PTC lead to activation of MAPK/ERK pathway which is a common carcinogenic pathway. Follicular Thyroid Carcinoma (FTC) is the second most common type of thyroid cancer. Just as PTC, it forms in follicular cells in the thyroid. It is either associated with chromosomal translocation or mutation in RAS subfamily oncogene. Medullary Thyroid Carcinoma (MTC) develops from the C (Calcitonin) cells in the thyroid. It is considered to be the third most common type of thyroid cancer, accounts for 3%, raised from RET proto-oncogene mutation.

Materials and methods

1. Cell culture

Normal rat thyroid cell line FRTL-5 cells were obtained from ATCC (American Type Cell Culture).The cells were grown at least 7 days in Coon's medium (5% calf serum/5 hormone mixture) supplemented with iodine (NaI at 10^{-8}M). Six hours before irradiation (0.1, 0.5 and 3Gy of X-rays), the cells were iodide deprived by medium replacement .Preparation of the medium is shown in table 1.

100 ml	**Product (stock concentration)**	**Final concentration**
91.3ml	Coon's medium	
5ml	NCS or FBS	5%
1ml	Peni-strepto	100U/ml
1ml	Fungizone	2.5µg/ml
1ml	Glutamin200mM	2mM
100µl	Insulin10mg/ml	10µg/ml
50µl	Transferrin10mg/ml	5µg/ml
7.2µl	Hydrocortisone 50µg/ml	3.6ng/ml
20µl	Glycyl-histidine-lysine 50µg/ml	10ng/ml
20µl	Somatostatin50µg/ml	10ng/ml
500µl	TSH 1 U/ml	5mU/ml

Table 1: composition of the medium forFRTL-5cell culture.

Samples were harvested at 6, 24, 48 and 72 hours post co-treatment. The study design is shown in figure 2.

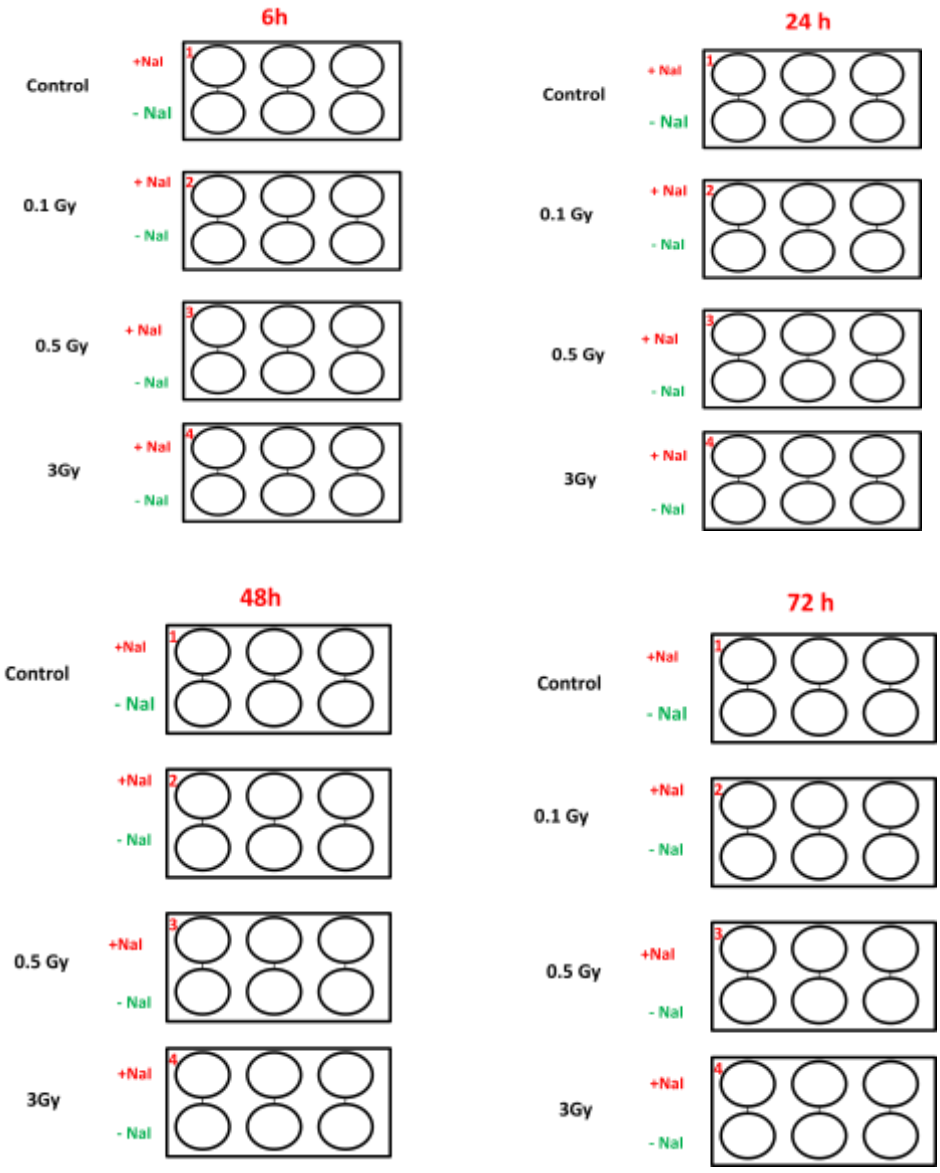


Figure 2: Experimental design. Two days before the co-treatment (irradiation and iodine deficiency), the cells were transferred in a 6 well plate at a density of $2X10^5$ cells/well. At the appropriate time before the co-treatment, medium was refreshed for all the wells. In each plate, the three upper wells were refreshed with medium containing NaI (Iodide) and the three lower wells with medium without NaI (ID). The cells in plate 1 are the controls, the cells in plate 2,3,4 are treated with irradiation at 0.1, 0.5 and 3 Gy and harvested 6, 24, 48 and 72 hours post co-treatment.

2. Cell counting

Cell counting was performed to calculate the number of cells to be seeded in each well plate. This has been done by Moxi Z automated cell counter that measures the number of the cells in 1 ml. $5x10^6$ cells were used to perform the Annexin V/ PI assay.

3. Annexin V/ Propidium Iodide (PI) assay

Apoptosis is associated with changes in the plasma membrane. In normal cells, phosphatidylserine (PS) molecules are located at the inner side of the plasma membrane, while during apoptosis these molecules are turned at the outer side of the plasma membrane so they can be labeled with the Annexin V-FITC conjugated antibody in the presence of calcium. In apoptosis, the cell membrane remains intact and therefore the propidium iodide (PI) cannot enter the cells. Due to their membrane properties, apoptotic cells will be recognized as the Annexin V-positive and PI-negative population when using flow cytometry for the analysis.

At the opposite, necrotic or dead cells lose the integrity of their plasma membrane and therefore will be positively stained with both PI and Annexin V. The living cells have an intact membrane and therefore will be recognized as the AnnexinV- negative and PI-negative population.

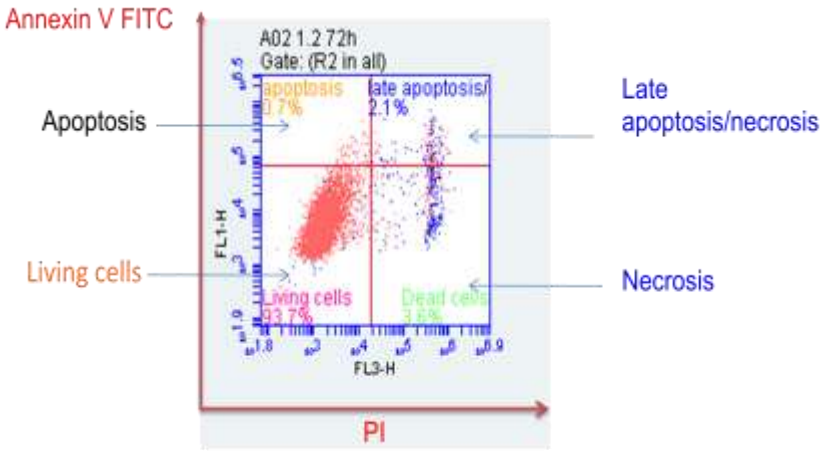


Figure 3: Different populations differentiated by Annexin V/ PI assay. Living healthy population is Annexin V-negative PI-negative (lower left quadrant), Apoptotic population is Annexin V-positive PI-negative (upper left quadrant), Late apoptotic/necrotic population is Annexin V-positive PI-positive (upper right quadrant) and population with aspecific membrane damage that is considered to be necrotic or dread population is Annexin V-negative PI-positive (Lower right quadrant).

In this study, Annexin V/ PI assay was performed to assess the number of apoptotic cells as a result of the co-treatment with radiations and iodine deficiency. FRTL-5 cells were stained with Annexin V-FITC and PI and then living cells, early apoptotic and late apoptotic/necrotic cells were assessed by flow cytometry according to the manufacturer's protocol (eBioscience company, see: supplement section A).Briefly, 2-5 × 10^5cells /ml were washed by PBs and then re-suspended in a binding buffer. Cells were stained with 5 μL of Annexin V-FITC to 195μL cell suspension, then incubated for 10 min at room temperature in the dark. Cells were washed with 200 μL of binding buffer, re-suspended in 190 μL binding buffer and then PI stained.

Both living cells, early apoptotic and late apoptotic/necrotic cells were determined using BD AccuriC6 flow cytometer. Color compensation for the Annexin V and PI staining was performed before the analysis. Cells treated with staurosporin which induces apoptosis were used as a positive control.

4. Western blot assay

Western blot was performed for protein detection. Protein extraction was carried out using " Ready Prep" extraction buffer from "Biorad" according to the manufacturer's protocol. Protein concentration was estimated using the BCA (bicinchoninic acid assay) method. Samples supplemented with Laemmli buffer and beta-mercaptoethanol were heated at 95°C for 5 minutes. Electrophoresis was performed on Sodium Dodecyl Sulfate (SDS)–polyacrylamide ready to use gels. The gels were pre-runed with a running buffer (contain 1% SDS) for 12 minutes at 160 volt. 5 minutes prior the end of the pre-run, samples were heated at 95° C to denature proteins, then runed along with a molecular weight marker on the gel for one hour.

The separated proteins were transferred onto a nitrocellulose membrane using the iBlotdry transfer system from Invitrogen. The membrane was blocked with a blocking buffer (contains sodium azide) for 1 hour at room temperature then it was washed two times/5-minwith a washing buffer (0.5 M Tris, 1.5 M Nacl, 0.5 % Tween20, PH 7.4). The primary antibody was added to the membrane (B-actin diluted 1/500, NF-KB diluted 1/200, pBAD diluted 1/200, pIKKa diluted 1/200 and AKT diluted 1/200) and incubated overnight at 4 °C.

The membrane was washed twice/5-minthen incubated with the secondary antibody (goat anti-mouse or anti-rabbit immunoglobulin G, Santa Cruz: 1/2000 in 8 ml washing buffer) for 45 min at room temperature, followed by 2 washes/5-min.

For visualizing the protein-antibody complex, the Qdot streptavidin conjugate (Santa Cruz) was used with a dilution of 1/2000 in 8 ml blocking buffer, and incubated with the membrane for 45 min under stirring. The membrane was then exposed to UV light and the bands were visualized using quantity one software (Biorad).

5. Statistical analysis

For the analysis of the living, apoptotic and dead populations, statistical analysis was performed using GraphpadPrism5 software. Results are presented as mean ± Standard Error of the Mean (SEM). Statistical differences between control and co-treated groups were determined by the ANOVA and post hoc tests and the results considered statistically significant when $P < 0.05$.

Results

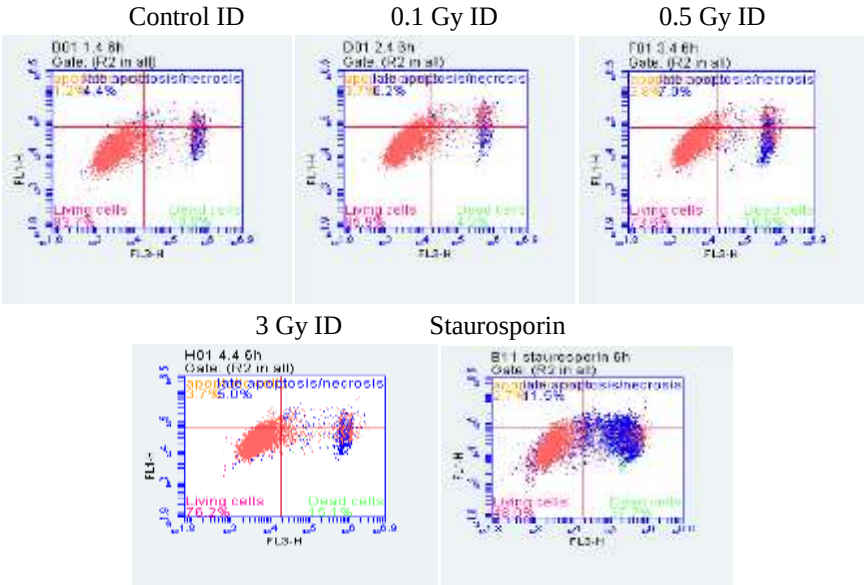
1- Co-treated cells show a resistance profile toapoptosis

Six hours after the co-treatment, annexinV/PI double staining was performed to evaluate the proportion of living, apoptotic, late apoptotic/dead cells using flow cytometry.

As shown in the figures underneath, the largest population is represented by the living cells. There was only little apoptotic cells and there count didn't increase after the co-treatment. The number of dead cells was also low. However, there is a little increase in the dead cells with the co-treatment especially in 0.5 Gy iodine deficiency and 3 Gy iodine deficiency conditions compared to control iodine deficiency, control iodine, 0.5Gy iodine and 3Gy iodine. Staurosprin, used in research to induce apoptosis by activating caspase-3 pathway, was used as a positive control. Six hours post-treatment with staurosporin showed that the cells went from early apoptosis to late apoptotic/necrotic and dead cells.

A

Annexin V/PI flow cytometry results



B

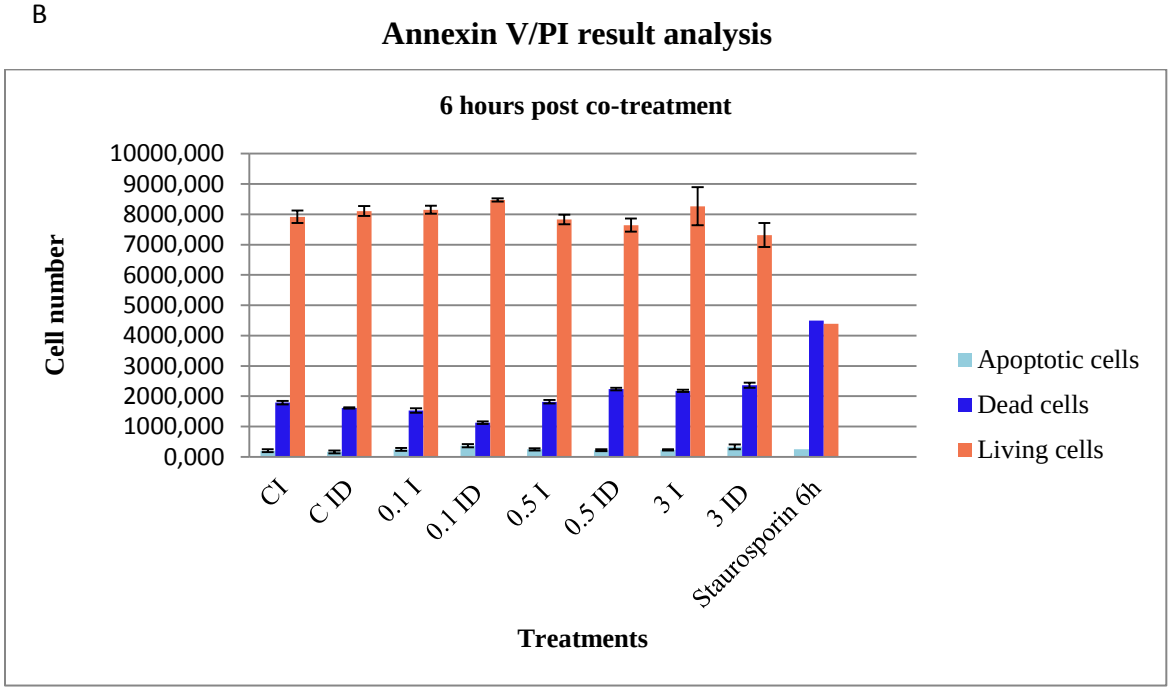
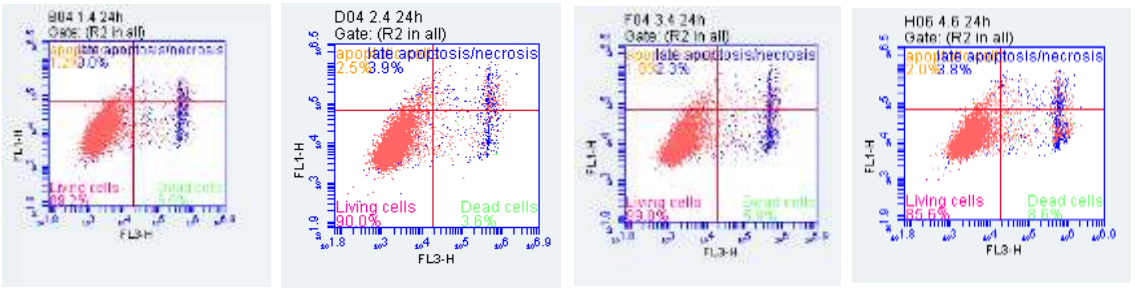


Figure 4: AnnexinV/PI double staining scatterplots. FRTL-5 cells were double stained with AnnexinV/PI to discriminate between living, apoptotic and late apoptotic/dead cells. A sctatterplot example for the condition without iodide is depicted above (A) showing the changes in the different cellular populations 6 hours post co-treatments. Staurosporin was used as a positive control. The scatterplots show that the fraction of the apoptotic cells remains unchanged at different doses of radiation while the fraction of the dead cells increases with increasing doses of radiation. Analysis of the results is shown in B. C I:control with iodide ,C ID: control without iodide.

For the co-treated cells that were harvested after 24, 48 and 72 hours, flow cytometry results showed the same profile as the co-treated cells that were harvested after 6 hours. The main population remained the living cells. No difference in the number of apoptotic cells was observed between all the treatments while a little increase in the number of the dead cells especially in 0.5Gy ID and 3Gy ID compared to control ID, control iodine and 0.5Gy iodine was noticed across the treatments as shown in figure 5,6 and 7.

Annexin V/PI flow cytometry results

Control ID | 0.1 Gy ID | 0.5 Gy ID | 3Gy ID



Annexin V/PI result analysis

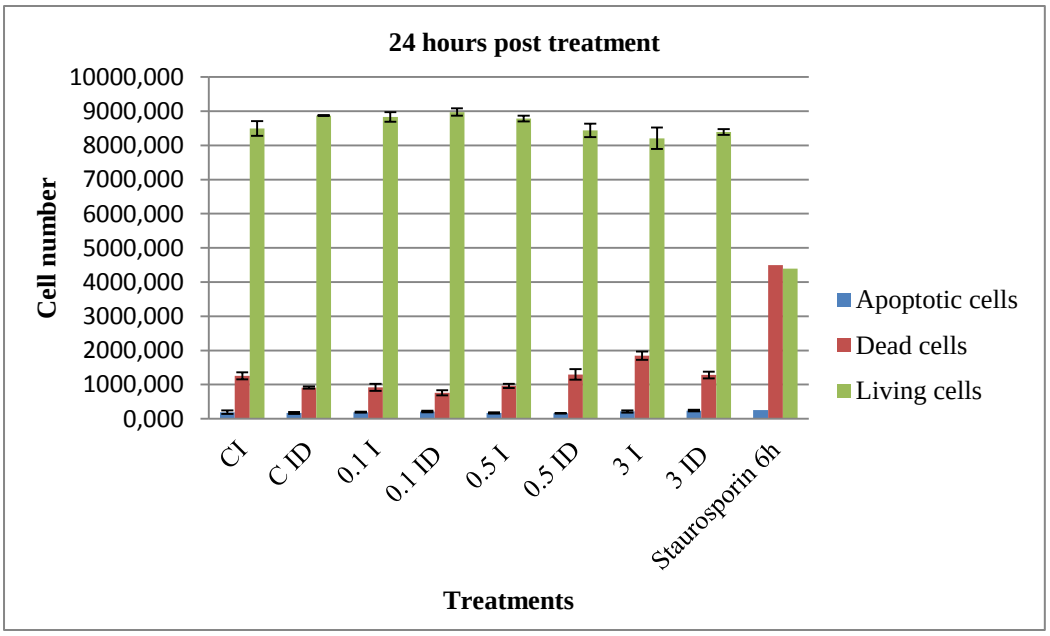
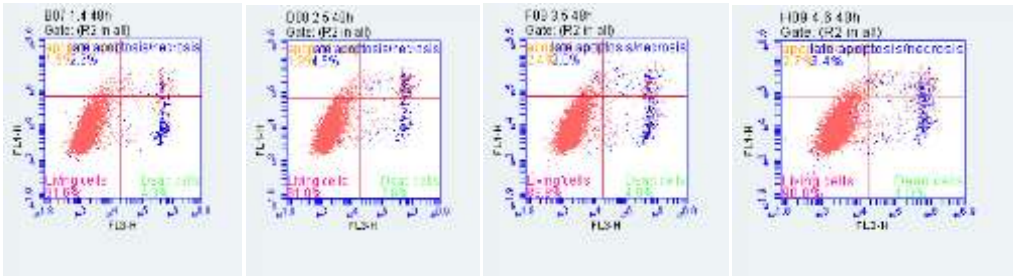


Figure 5: Scatterplots and analysis of the co-treated cells with different doses of radiations and iodine deficiency that were harvested after 24 hours and compared to the controls.

Annexin V/PI flow cytometry results

Control ID 0.1 Gy ID 0.5 Gy ID 3Gy ID



Annexin V/PI result analysis

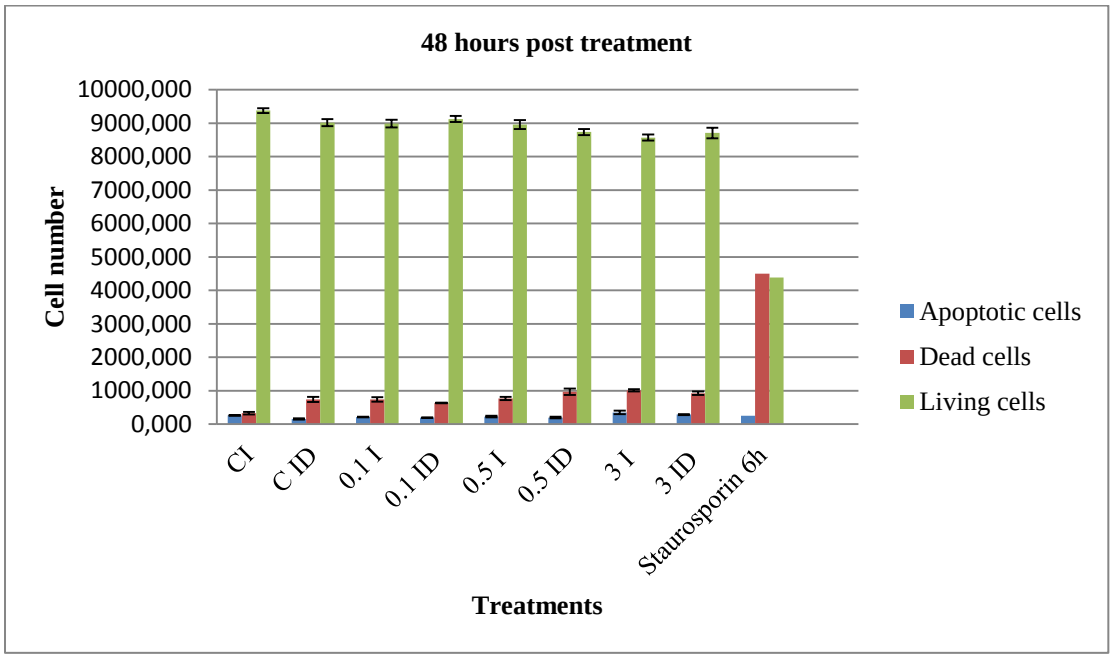


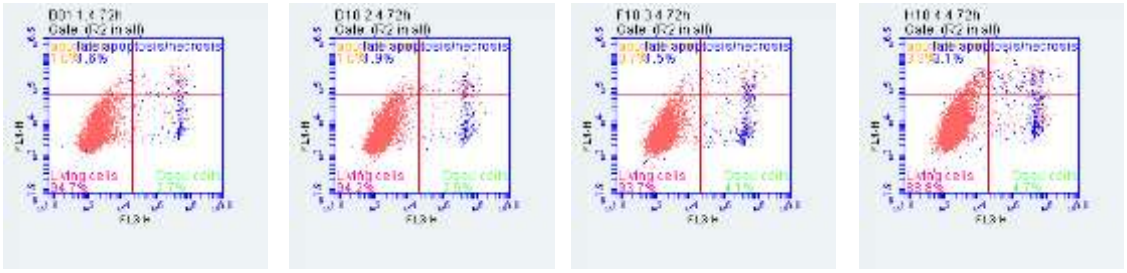
Figure 6: Scatterplots and analysis of the co-treated cells with different doses of radiations and iodine deficiency that were harvested after 48hours and compared to the controls.

As shown in figure 4,5,6 and 7, no significant differences in the number of apoptotic cells were observed between all the treatment and that over the different time points of the experiment. The number of apoptotic cells was very low as shown in the upper left quadrant (Annexin V-positive PI-negative).

The dead cells located at the upper and lower right quadrant(Annexin V-negative PI-positive and Annexin V-positive PI-positive),showed the same profile in all the treatment and at different time points, meaning that they were low in number but a little increase with the dose was observed especially in 0.5 Gy ID and 3Gy ID compared to Control ID, control iodine, 0.5Gy iodine and 3Gy iodine.

Annexin V/PI flow cytometry results

Control ID 0.1 Gy ID 0.5 Gy ID 3Gy ID



Annexin V/PI assay result analysis

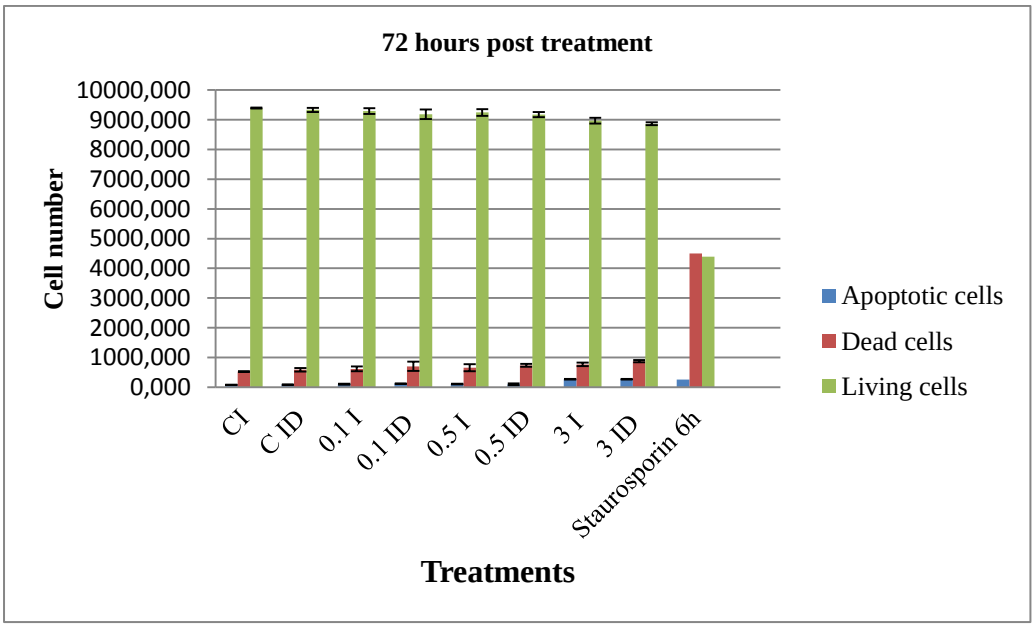


Figure 7: Scatterplots and analysis of the co-treated cells with different doses of radiations and iodine deficiency that were harvested after 72 hours and compared to the controls.

These data suggest that there must be molecular mechanisms turned on/off that make the cells resistant to the co-treatment. Of note, cancer may originate from a single transformed proliferating cell. Therefore, the fact that these cells seem to be resistant to apoptosis and do not die massively after high doses of radiation imply that they must have responded to the co-treatment with a survival mechanisms and this may allow them to transmit genetic damage to the next generation. To understand the mechanism of radio-resistance in these cells, we looked at the survival and apoptotic pathways by evaluating the expression level of different pro-survival and pro-apoptotic proteins using western blot.

2. Irradiation and iodine deficiency increase NF-KB p52 protein expression

NF-KB is a pro-survival transcription factor that regulates a great number of genes involved in cell cycle regulation and anti-apoptosis processes[27]. Thus, we looked at the possible involvement of this transcription factor in the radio-resistance profile of the FRTL-5 cells in the presence or absence of iodide.NF-KB p52expression level was evaluated in the FRTL-5 cells 6 hours post co-treatment and the experiment was performed on 3 biological replicates.

As shown underneath in figure 8A,B and C, NF-KB p52 was expressed in the control cells at a basal level which increased in a dose dependent manner (0.5 Gy and 3Gy). However, the NF-KBp52 level was higher in the cells that were cultured without iodide and here again NF-KBp52 expression followed a doses dependent increase.

In this experiment, Hela whole cell lysate and RET/PTC tissues extracts from mouse thyroids with papillary cancer were used as positive control for NFkBp52 expression.

These results indicate that NF-KB activation is turned on in the FRTL-5 cells after the co-treatment, and possibly there is a synergistic effect of the co-treatment on NF-KBp52 activation. These results will be confirmed by repeating the experiment on replicate 2 because of the fade bands. Thereafter, densitometric analysis of the bands will be possible and the values obtained will be normalized against the ones from B-actin.

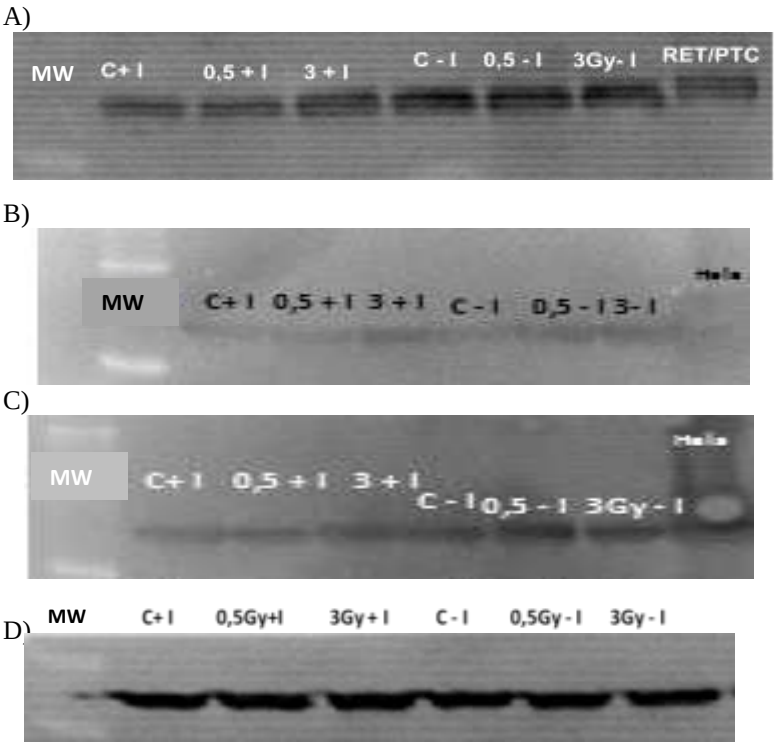


Figure 8. NF-KB p52protein expression is increased with the co-treatment. NF-KBp52 protein expression was evaluated in FRTL-5. MW is the Molecular Weight. C+I: control with iodide, C-I: control without iodide, 0,5 /3 +I: irradiated cells with 0,5 or 3 Gy with iodide, 0,5/ 3 -I: irradiated cells with 0,5 or 3 Gy without iodide, A) is replicate 1, B) is replicate 2 and C) is replicate 3. D) is B-actin.

3. Irradiation and iodine deficiency increase pIKKaprotein expression

IKKa is a protein kinase which is upstream of NF-KB p52 activation. Upon stimulation with ionizing radiation, TNF, lipopolysacharide or interlukin, phophorylation of Ser-32 and Ser-36 on IKKa occurs and activation of IKKatake place[27].

Since NF-KB p52 expression level was found to be increased with the co-treatment, we looked at pIKKa expression level in cells exposed to the co-treatment with irradiations and iodine deficiency and harvested after 6 hours via western blot assay. A431 (epidermoid carcinoma) cells were used as the positive control.

As expected, pIkka expression level was increased with the irradiation dose and was increased even more with the co-treatment as shown in figure 9. Thus, possibly there is a synergistic effect of the co-treatment on pIkka activation as well. This has been done for replicate 1, it needs to be evaluated for triplicate 2 and 3 as well.

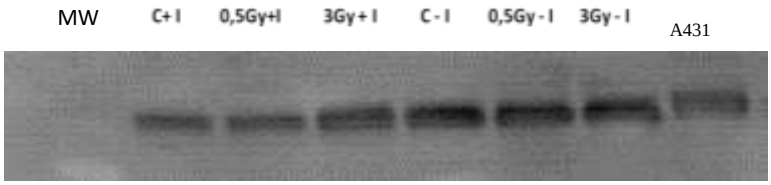


Figure 9:pIKKa expression level is increased with the co-treatment. pIKKa protein expression was evaluated with western blot in FRTL-5. MW is the Molecular Weight. C+I : control with iodide, C-I: control without iodide, 0,5 /3 +I: irradiated cells with 0,5 or 3 Gy with iodide, 0,5/ 3 -I: irradiated cells with 0,5 or 3 Gy without iodide.

4. AKT-1 and pAKT expression levels

Akt is a protein which acts downstream of the PI3K pathway which is a cell survival pathway.PI3K/Akt pathway is involved in the indirect NF-KB activation[27]. Activation of Akt1 takes place by phosphorylation of Thr308 and Ser473, upon phosphorylation, it activates many proteins that affect cell growth, cell cycle and cell survival

We evaluated Akt-1 expression level in the co-treated cells that were harvested after 6 hours in replicate 3 by western blotting. Hela whole cell lysate was used as a positive control.

pAkt-1 expression level was evaluated in replicate 1.As shown in figure 10A, the expression level of Akt-1 was low in all the treatment groups, while pAkt-1 level as shown in figure 10Bwas increased with the irradiation dose and increased even more with the co-treatment to reach the highest level in cells treated with irradiation at 3Gy and iodine deficiency. So, the co-treatment may activate pAkt-1 which is also pro-survival protein.

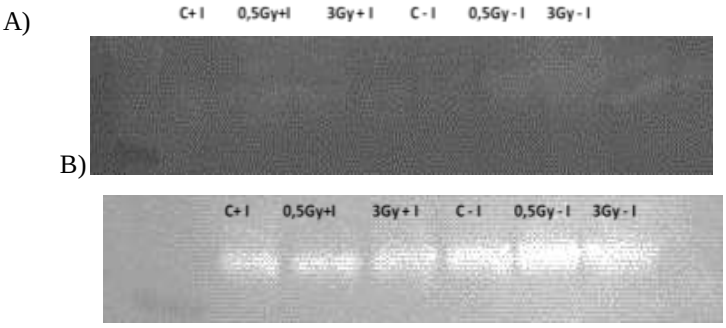


Figure 10: Akt-1 and pAkt-1 expression levels. Akt-1 and pAkt-1 expression levels were evaluated in the co-treatment conditions and compared to the control cells. A) Akt-1 level in replicate 3. B) pAkt-1 level in replicate 1.

5. The pro-apoptotic protein Bad and pBad expression levels don't change with the co-treatment.

The expression levels of the pro-apoptotic Bcl-2-Associated Death promoter (BAD) protein and its phosphorylated form were evaluated to assess their activation as a result of the co-treatment with radiations and iodine deficiency. Bad is a member of the Bcl-2 gene family that is important for apoptosis, when activated it initiates apoptosis by activating the pro-apoptotic caspase cascade.

Bad and pBad expression levels were evaluated in the co-treated cells replicate 2 and 3 that were harvested after 6 hours. The co-treated cells didn't show any expression for Bad nor pBad as shown in figure 11. The results suggest that both Bad and its phosphorylated form are not activated as a result of the co-treatment .

This result support the previous findings of flow cytometry, as the apoptotic population were very low in number in the co-treated cells.

Figure 11: Expression level of pBad in the FRTL-5cells harvested 6 hours after the co-treatment.

For all the western blot results, to calculate the densitometric values, B-actin expression level need to be done for the 3 replicates.

Discussion

In this study we investigated the combined effect of radiations and iodine deficiency on thyroid cells. Our data suggested that FRTL-5 co-treated cells show a radioresistance profile that is explained by activated pro-survival molecules, most importantly NF-κB.

The transcription factor NF-κB initiates a pro-survival network and can activate a great number of genes that are involved in stress response. There are a diverse external stimuli that can activate NF-KB and its downstream genes. For instance, bacterial and fungal infection, inflammation, ionizing radiation and oxidative stress, can all activate NF-KB. There are two pathways for NF-KB activation. The classical pathway which is responsible for IκB-α, IκB-β, and IκB-ε rapid degradation and referred to as IKK-β-dependent pathway, and the alternative pathway that lead to the processing of p100 and activation of p52/RelB and referred to as IKK-α-dependent pathway. The alternative pathway can be triggered by TNF receptor family activation. After the activation of NF-KB, it translocates to the nucleus to regulate the transcription of various genes that are necessary for an enhanced cell survival. The anti-apoptotic function of NF-KB is due to TNF-induced NF-KB activation that result in transcription of anti-apoptotic genes such as c-IAP-1 and c-IAP-2, these molecules inhibit apoptosis by blocking the caspase function. In addition to that, NF-KB has the ability to regulate the cell cycle by induction of G1/S protein cyclinD1 that is important for cell cycle G1/S transition. There are several studies reported that over expression of HER2 also activates NF-KB and increase cell proliferation and survival. PI3K/AKT pathway which is another cell survival pathway is involved in HER2 activation, thus possibly involved in NF-KB activation.

Ionizing radiation can activate NF-KB via a direct or an indirect pathway. Ionizing radiations cause direct DNA damage via single stand breaks or double stand breaks. This lead to the activation of nuclear ATM protein which translocates to the cytoplasm and activates NF-KB through regulation of IKK activity. Ionizing radiation can also activate

NF-KB through the production of ROS which can induce DNA damage and activate NF-KB via the TRAFs pathway as well and this explains the radioadpative resistance of the cells treated with ionizing radiations. Iodine deficiency can also induce ROS production, thus this can activate NF-KB and upregulate the effector genes network that are regulated by it. Therefore, the co-treatment of iodine deficiency and radiations may have a synergistic effect on thyroid cells.

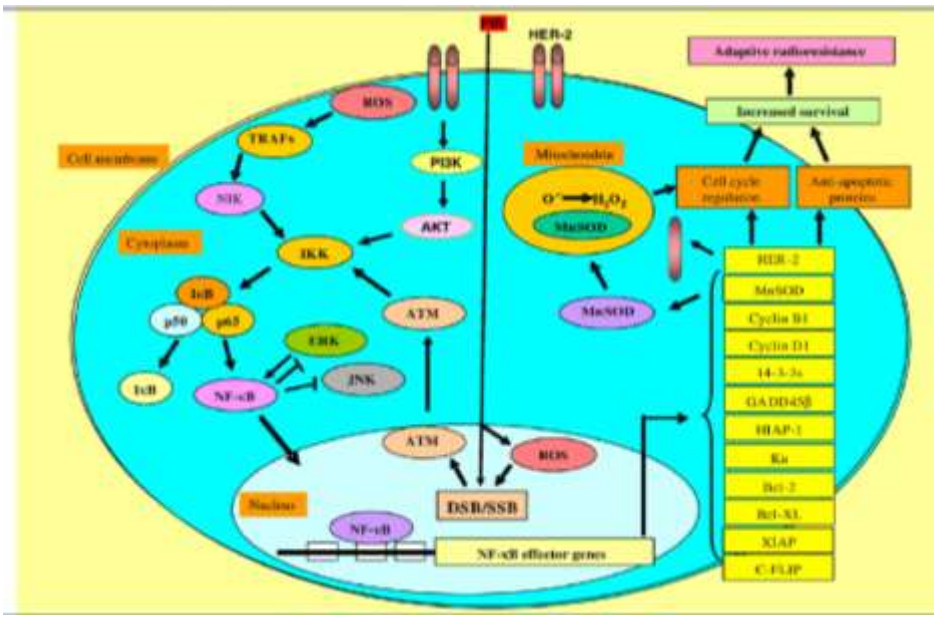


Fig. 12. Schematic representation of the NF-κB signaling network*(Ahmed, and Li, 2008)*.

From the preliminary previous results, the combined effect of irradiation and iodine deficiency probably lead to up regulation of pro-survival proteins and down regulation of pro-apoptotic proteins. Activation of pro-survival pathways that increase cell survival is from the characteristics of cancer cells, so irradiation and iodine deficiency might be a risk factor to thyroid cancer.

Moreover, knowing the existence of thyroid microcarcinoma that range from 3-36% at autopsies[31, 32], the exposure to radiations and iodine deficiency might be considered as a risk factor to turn this silent thyroid microcarcinoma to real thyroid cancer.

Further research is needed to understand the exact mechanisms involved in the combined effect of radiations and iodine deficiency and to evaluate whether these two factors represent a risk for thyroid cancer.

Conclusion and synthesis

Thyroid cancer is one of the malignancies that are induced by radiation exposure as shown by epidemiological studies performed after the Chernobyl accident. Unlike the effect of high doses of radiation which is known from epidemiological evidences, the effects of low doses of radiations are still unclear. Besides radiations, 2 billion people are suffering from iodine deficiency worldwide. Radiation, iodine deficiency, age and genetic background are important determinants in thyroid cancer development.

However, there are little knowledge about the combined effect of low doses of external radiations (X-rays) and iodine deficiency on thyroid cells. Therefore, this research has been performed to understand the basic mechanisms of thyroid cancer risks after low doses of external radiations under iodine deficient conditions for a better prevention of the general population.

To investigate the combined effect of radiations and iodine deficiency on thyroid cells, FRTL-5 cell culture (rat thyroid cells) were treated with different doses of radiations(0.1, 0.5 and 3 Gy) under iodine deficiency then harvested after 6,24,48 and 72 hours.

Evaluation of the fraction of apoptosis/necrosis in the co-treated cells through Annexin V/PI assay, showed that the co-treated cells exhibit a resistant profile to apoptosis. The apoptotic population was very low in count and there were no differences between apoptotic cell counts over all the treatments at different time points. The dead cells were also low in count, however a small increase was observed with increasing doses. The same observations were made for all the time points under study. These outcomes suggest that the co-treatment affect the molecular mechanisms inside the cells that make them resistant to apoptosis.

To investigate the different pathways that are involved in the combined effect of radiations and iodine deficiency, the expression level of different proteins have been

evaluated using western blot assay. The over expression of NF-κBp52 proteinwith the co-treatment may explain the resistance of the co-treated cell to enter apoptosis. Since NF-KB regulates and is responsible for the transcription of a network of genes involved in anti-apoptosis and cell cycle regulation, it is possible that irradiation and iodine deficiency have a synergistic effect on NF-KB activation that may increase the cell survival and make the cells resistant to apoptosis. Previous studies on different cell lines including Hela (cervical cancer), 293 (Human epithelial embryonic kidney) and keratinocytes have shown that both ionizing and ultraviolet radiations activates the pro-survival factor NF-KB that was behind the radioadaptive resistance of these cell lines[26, 33, 34].

Our finding is further supported by the fact that pIKKa, the upstream activator of NF-KB p52 was also found increased with the co-treatment. In addition, pAKT another prosurvival molecule belonging to the PI3K pathway and involved in NF-KB activation was also found increased with the co-treatment.

These results support the study hypothesis, however further research is needed to investigate more in depth the mechanisms behind the radioresistance of the FRTL-5 cells to radiations under iodine deficiency.

The future perspectives of this study include further investigation of the pro-survival and pro/anti-apoptotic genes/proteins, evaluation of the ROS production with the co-treatment and microarray analysis to have a global view on the molecular mechanisms that are switched on/off.

In summary, the exposure to radiations and iodine deficiency may have a synergistic effect on the activation of pro-survival pathways in thyroid cells. Therefore, radiations and iodine deficiency might be considered as risk factors for thyroid cancer. This study is important for radioprotection regulation, especially for those patients receiving high doses of radiation for therapeutic reasons.

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Supplements:

1. Annexin V-FITC/PI assay (Apoptosis detection Kit)

- **Principle of the Test**

Annexin V exhibits anti-phospholipase activity and binds to phosphatidylserine in the presence of calcium. FITClabelling allows simple direct detection of FITC positive cells by flow cytometry analysis. Counterstaining with propidium iodide allows the discrimination between apoptotic and necrotic cells.

- **Preparation of Reagents**

Dilute Binding Buffer (4x) 1:4 in distilled water (50 ml binding buffer and 150 ml distilled water).

- **Test Protocol**

a. Wash cells in PBS by gentle shaking or pipetting up and down.

b. Resuspend cells in 200 µl Binding Buffer (1x); cell density should

be 2-5x105/ml.

c. Add 5 µl Annexin V-FITC to 195 µl cell suspension.

d. Mix and incubate for 10 min at room temperature.

e. Wash cells with 200 µl Binding Buffer (1x) and resuspend in 190 µl

Binding buffer (1x).

f. Add 10 µl Propidium Iodide (20 µg/ml)

g. Perform FACS analysis.