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QUERCETIN DEVELOPS SENSITIVITY OF ADENOCARCINOMA CELLS TO RADIATION *VIA* LOWERING METASTASIS AND PROMOTING CASPASE-3 INDEPENDENT CELL DEATH

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ABSTRACT: Quercetin (QCN) is a flavonoid available in fruits and vegetables, and it has shown several medicinal properties, including anticancer properties. However, the radiosensitizing properties of QCN on adenocarcinoma cells have not been greatly looked into yet. Thus in the present study, we investigate the effect QCN would have on the lung adenocarcinoma cell line NCI-H23 (H23) when QCN is combined with radiation. We checked for QCN's effect on cell survival of H23 and the embryonic HEK293T cells. We checked the effect that QCN alone and QCN in combination with radiation would have on oxidative stress, metastatic proteins MMP-2 and p21, the expression of AKT1 and PTEN genes, and the apoptosis-associated genes in H23 cells. We observed that QCN alone and in combination with radiation lowered the cell survival of H23 cells while not affecting the cell survival of HEK293T cells. The QCN treated groups showed a spike in oxidative stress, lowering of the levels of MMP-2 and p21 proteins, up-regulation of PTEN gene and down-regulation of AKT1 gene expression, and a caspase-3 independent cell death. From our results, we can state that QCN had a radiosensitizing effect on H23 cells, and it achieved so *via* lowering metastatic proteins, lowering AKT1 expression, promoting PTEN expression, and promoting caspase-3 independent cell death.

INTRODUCTION: Adenocarcinoma is a cancer of the glandular cells that are involved in secreting various substances [1]. Adenocarcinomas can develop in different organs like the lungs; stomach, prostate, *etc.* [2, 3, 4] Pulmonary adenocarcinomas deserve a special mention as they happen to be amongst the most common of all types of cancers and also have a very high rate of mortality [2].

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Pulmonary adenocarcinoma is a histologic subtype of non-small cell lung cancer [2]. The lung-based adenocarcinomas are highly metastatic and can spread *via* the lymph nodes and blood vessels to all the major organs of the body [5].

Metastasis allows the cancer cells to escape the immune system, and also cancers modulate the immune system to promote metastasis [6]. Another aspect of metastasis is that it is involved in bolstering up the resistance of cancer cells towards therapeutic interventions [7]. This above fact is indicated by the lowly 5-year survival rate of less than 5% in the case of patients with metastatic lung adenocarcinomas [8]. Some of the proteins critical for metastasis to occur are p21 and matrix metallo-

proteinase-2 (MMP-2) [9, 10]. p21 participates in promoting epithelial to mesenchymal transition (EMT) and stem cell renewal, which are critical for cancer progression and metastasis [11]. p21 drives resistance of cancer cells to chemotherapy and radiation [11] while inhibiting apoptosis [9]. MMP-2 is critical for initiating EMT and also for its more traditional roles of promoting metastasis and angiogenesis; both of which are the major hallmarks of cancer [12].

MMP-2 is also involved in promoting cell proliferation, inhibiting apoptosis, and driving resistance of cancer cells to treatment procedures [13]. Another component in the midst of p21 and MMP-2 is the protein kinase b (Akt) protein. Akt is involved in promoting the oncogenic activities of p21 [14]. Akt has also been reported to be involved in signaling processes that ultimately lead to the expression of several metastatic proteins, including MMP-2 [15].

Phosphatase and Tensin homologue (PTEN) is a tumour suppressor protein that acts as an antagonist to all the Akt mediated actions by inhibiting the phosphoinositide 3 kinase (PI3K) mediated activation of Akt [16]. Metastatic adenocarcinomas have high levels of p21, MMP-2 and Akt proteins which correlated with the dismal survival rates of patients with lung adenocarcinomas [14, 17].

Another implication of the low survival rates is the lack of effectiveness of the current cisplatin-based chemoradiotherapy treatment protocol against pulmonary adenocarcinoma [8]. Inhibitors for Akt, MMP-2, and p21 have shown severe side effects in clinical trials [18, 19, 20,] and thus there is an urgent need to check for phytochemicals that can act against these pre-cancerous proteins while having minimal side effects. In this study, we investigated the role of the flavonoid quercetin in promoting sensitization in the lung adenocarcinoma cell line H23 towards radiation and the effect QCN would have on the metastatic proteins MMP-2 and p21 levels. QCN is a flavonoid available in a large number of vegetables and fruits which have been consumed by humans [21]. QCN has many several medicinal properties, including anti-cancer [21] properties, but its radiosensitizing ability on lung adenocarcinoma has not received much attention, and as such, we embarked upon this study.

MATERIALS AND METHODS: For carrying out the study, QCN was purchased from HiMedia (India). QCN stock solution was prepared in dimethylsulphoxide (DMSO), and the working solution was made in RPMI 1640/DMEM by diluting the stock solution in the respective media. The working stock had a DMSO concentration of 0.01%.

Cell Culturing: H23 cancer cell line and HEK293T embryonic cell line was obtained from National Centre of Cell Science, Pune, India. H23 cells were cultured in an RPMI 1640 (Invitrogen, USA) based media containing 2 mM L-glutamine (Invitrogen, USA), 10% fetal bovine serum (FBS) (Himedia, India). HEK293T embryonic cell line was grown in a media consisting of DMEM (Invitrogen, USA), 1% non-essential amino acids (Invitrogen, USA), 2 mM L-glutamine, and 10% FBS. An antibiotic mixture containing penicillin, streptomycin, and neomycin (Invitrogen, USA) was added at a concentration of 0.1%.

Proliferation Assay: H23 cells were divided into three different sets of treatment groups: treated with QCN alone, treated with X-radiation alone and, treatment with QCN followed by treatment with X-radiation (combination groups). X-radiation was administered at a dose rate of 1 Gy/min for total doses of 2, 4, 6, and 8 Gy, respectively. A group of cells were sham-treated and served as the control. Cells were treated with QCN for 24 h in the case of the QCN containing groups, while the control and radiation alone groups were treated with RPMI + 0.01% DMSO. After 24 h the cells in the radiation alone group and combination group were subjected to X-irradiation (Faxitron, USA). Thereafter the cells from all the groups were incubated in fresh media for 24 h. Once the treatment period was over, 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) solution was added, and the absorbance readings were taken at 595 nm using a multiwell plate reader (Biorad, USA).

HEK293T Cell Viability Assay: HEK293T cells were grown on a 6-well plate with each well containing 2×10^5 cells. The treatment protocol for the HEK293T cells was the same as that for the H23 cancer cells. The cells were detached from the growing surface by treatment with trypsin, and then

a cell count of the trypan blue-stained viable cells was performed. The cells were viewed in an inverted microscope, and the viable cells within the chambered regions of the haemocytometer (HBG, Germany) were counted.

H23 Cell Viability Assay: Observing the outcomes of the above experiments, we proceeded with the rest of the study by limiting the radiation dose to a single dose of 6 Gy in both the radiation-treated groups. However, the treatment protocol for each of the individual groups was kept identical to the proliferation assay. For taking a trypan blue count of the viable and dead cells, the same protocol as that for the HEK293T cells was followed. Cells with clear cytoplasm and a ring of blue stain on the membrane were accounted for as viable and cells with a blue cytoplasm and a distorted membrane were counted as dead [22].

Clonogenic Assay: We performed the clonogenic assay by making minor modifications to the protocol communicated by Crawley *et al.,* (2016) [23]. Once the treatment process of the cells was over the cells were detached from the surface by treatment with trypsin and were transferred to a 6-well plate at a per well density of 2×10^5 cells and grown for 2 weeks. At the end of 2 weeks, cell fixation was achieved via methanol, proceeded by crystal violet staining. The visible colonies were counted.

Protein Carbonylation Assay: For checking the levels of carbonylated proteins in the cells, we followed the protocol mentioned by Colombo *et al.,* (2016) [24] with minor modifications. We performed the same treatment protocol followed by the addition of 10 mM 2, 4-dinitrophenylhydrazine (DNPH) to the cell lysates. The cell lysates were then washed with 20%, and 10 % trichloroacetic acid (TCA) succeeded by washing with an equimolar solution of ethyl acetate and ethanol. Subsequently, a solution of 6 M guanidine hydrochloride was used to dissolve the pellet, and the readings of this solution were taken at a wavelength of 366 nm using a spectrophotometer.

DEVDase Assay: The DEVDase assay was conducted as per the instructions of the manufacturer (ABCAM, UK) to evaluate the activity of caspase-3 in the treated samples. The cell lysates were incubated with acetyl-Asp-Glu-Val-Asp-para nitroaniline (DEVD-pNA) dissolved in substrate buffer for a period of 4 hr at 370C, and the amount of free pNA released by lysis of DEVD-pNA by caspase-3 was measured by spectrophotometric readings taken at 400 nm. Changes in caspase-3 activity were compared with the control set and expressed in terms of fold change.

Western Blotting Analysis: Western blotting was executed using a protocol standardized in our laboratory [25]. We loaded 30 μg of the protein lysate and separated the proteins using sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to a protein adsorbing membrane. The membranes were probed for MMP-2 and p21 proteins using specific antibodies (ABCAM, UK), and the protein detection was done using secondary antibodies conjugated with alkaline phosphatase. Alkaline phosphate yields a coloured product when reacted with its appropriate substrate allowing the detection of the protein of interest [25].

Gene Expression Analysis: For analyzing the mRNA expression levels of AKT serine/threonine kinase 1 gene (AKT1), CASPASE3, B-cell lymphoma 2 (BCL2), B-cell lymphoma 2 Extra Large (BCL-XL), Bcl2 Associated X protein (BAX), and Beclin-1 (BECN1) genes we used a reverse transcriptase based kit (Applied Biosystems, USA) to convert the RNA isolated using trizol reagent (Sigma, USA) into cDNA. The cDNA was then amplied using an SYBR Green kit (Applied Biosystems, USA) on a PCR machine with real-time detection systems (Applied Biosystems, USA). β-actin gene (ACTB) was used as the reference gene, and the difference in the gene expression levels of the target genes was quantified in relation to the reference gene [26].

Statistical Analysis: The data obtained was calculated and expressed in terms of mean ± standard deviation. For calculating the significance values, ANOVA was used (SPSS, USA), and values between groups were considered to be statistically significant if p values were less than 0.05.

RESULTS: QCN promotes sensitization of H23 cells to radiation: The first step in our study was to check the radiosensitizing ability of QCN upon the H23 cells. As Teh *et al.,* (2013) [27] had previously reported that the IC50 of QCN against the H23 cells stood at 58 µM, we conducted our study using the same dose of QCN. Treatment of the H23 cells with 58 µM of QCN alone lowered the survival of the H23 cells to approximately 55% (**p< 0.01). The percent survival of the H23 cells showed a gradual reduction in survival along with increasing doses of radiation. However, a 50% reduction in cell survival was observed only the combination groups involving 6 and 8 Gy of radiation **Fig. 1A** and **Table 1** (##p< 0.01). Upon checking the effect of QCN on the embryonic HEK293T cells **Fig. 1B** and **Table 2** both when used alone and in combination with radiation, we observed that QCN didn't cause any significant changes in cell viability except in the 6 Gy combination group where QCN offered a significant radioprotective effect upon the HEK293T cells as could be deduced from the higher number of viable cells in the QCN + 6 Gy group (approximately 85%) with respect to the 6 Gy alone group (approximately 70%) (##p<0.01).

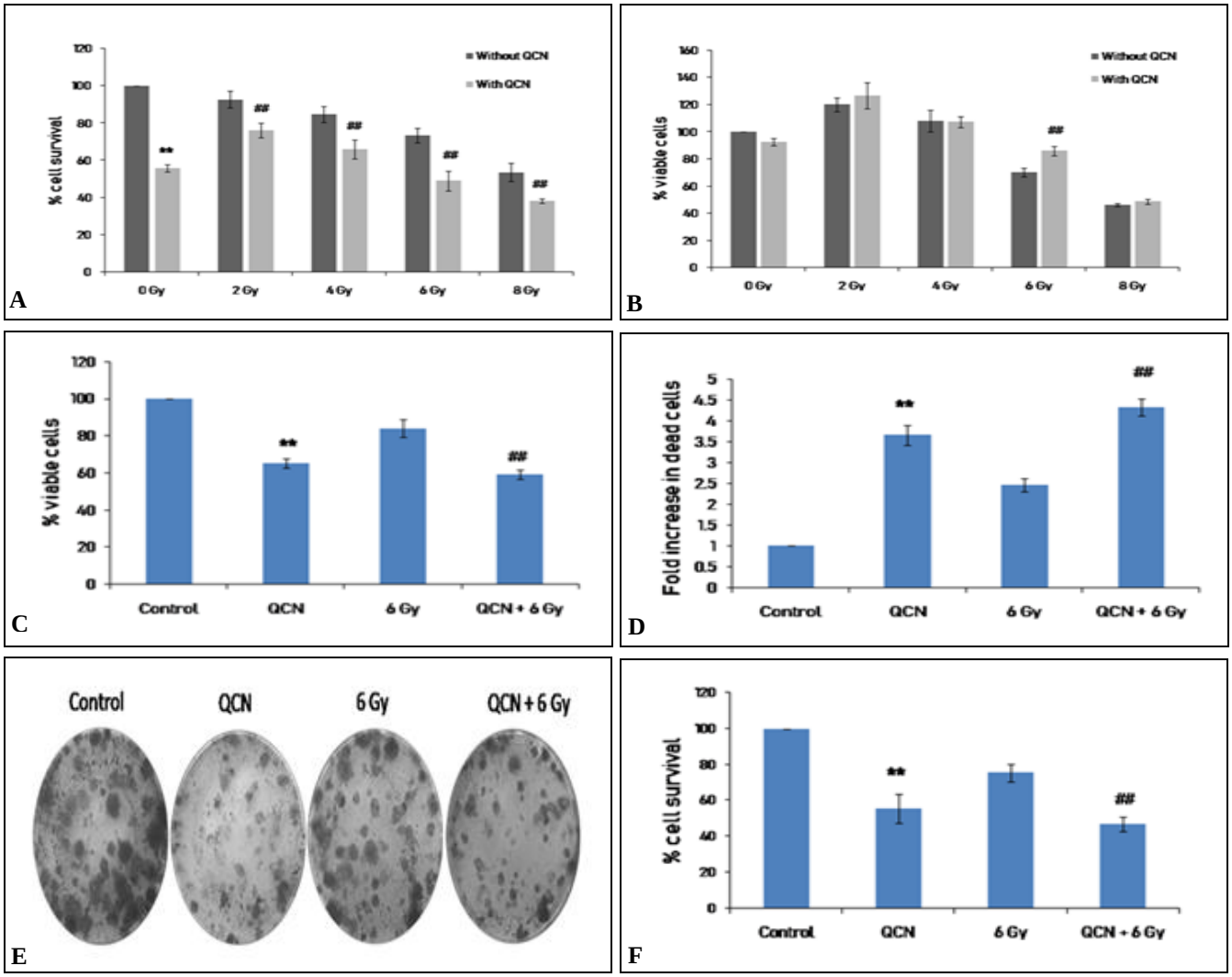

FIG. 1: QCN LOWERS H23 CELL SURVIVAL SINGULARLY AND IN COMBINATION WITH RADIATION. (A) GRAPH SHOWING PERCENT CANCER CELL SURVIVAL UNDER VARIOUS TREATMENT GROUPS. **P< 0.01 *vs.* CONTROL AND ##P < 0.01 *vs.* 2 GY, 4 GY, 6 GY AND 8 GY IRRADIATED GROUP. (B) BAR GRAPH FOR PERCENT CELL SURVIVAL OF NORMAL FIBROBLAST CELLS UNDER VARIOUS TREATMENT GROUPS. ##P< 0.01 *vs.* THE RESPECTIVE IRRADIATED GROUP. (C) BAR GRAPH SHOWING PERCENT VIABLE H23 CELLS AFTER VARIOUS TREATMENTS. **P< 0.01 *vs.* CONTROL AND ##P< 0.01 *vs.* 6 GY GROUP. (D) BAR GRAPH SHOWING FOLD CHANGE IN DEAD H23 CELLS AFTER VARIOUS TREATMENTS. **P< 0.001 VS. CONTROL AND ##P< 0.01 *vs.* 6 GY GROUP. (E) H23 COLONIES AFTER STAINING WITH CRYSTAL VIOLET. (F) BAR GRAPH SHOWING PERCENT SURVIVING CELLS FROM COLONY FORMING ASSAY. **p< 0.01 vs. control and ##p< 0.01 vs. 6 Gy. All values are expressed as mean ± standard deviation.

Since, we are investigating QCN as a safer alternative to current radiosensitizers, and as QCN offered a significant reduction in H23 cell survival in combination with 6 Gy while also offering radioprotective action at the same radiation dose, we proceeded ahead with the rest of the study using only the 6 Gy dose of radiation. QCN lowered the number of viable H23 cells in both the QCN treated groups (**p<0.01 and ##p<0.01) while increasing the dead cell count (**p<0.01 and ##p<0.01) as evident after taking a trypan blue cell count **Fig. 1C** and **1D**. We performed the colony-forming assay to further confirm the findings of the above studies, and we observed that the QCN treated groups showed a decrease in the number of colonies formed **Fig. 1E** and a subsequent lowered number of surviving cells **Fig. 1F** (**p<0.01 and ##p<0.01).

TABLE 1: TREATMENT PROTOCOL AND % CELL SURVIVAL OF H23 CELLS

Experiment performed	Treatment protocol		% cell survival
	QCN administered	Radiation dose (Gy)	
Proliferation assay	No	0	100 ± 0
	Yes	0	55.68 ± 1.79
	No	2	92.733 ± 4.54
	Yes	2	75.93 ± 3.89
	No	4	84.84 ± 4.3
	Yes	4	65.92 ± 4.97
	No	6	73.39 ± 3.98
	Yes	6	48.87 ± 5.33
	No	8	53.45 ± 4.89
	Yes	8	37.98 ± 1.22

TABLE 2: TREATMENT PROTOCOL AND % CELL SURVIVAL OF HEK293T CELLS

Experiment performed	Treatment protocol		% cell survival
	QCN administered	Radiation dose (Gy)	
HEK293T cell viability assay	No	0	100 ± 0
	Yes	0	92.45 ± 2.6
	No	2	119.92 ± 4.83
	Yes	2	126.65 ± 9.67
	No	4	107.83 ± 7.9
	Yes	4	107.24 ± 3.93
	No	6	70.067 ± 3.11
	Yes	6	85.68 ± 3.54
	No	8	46.11 ± 1.08
	Yes	8	48.54 ± 1.82

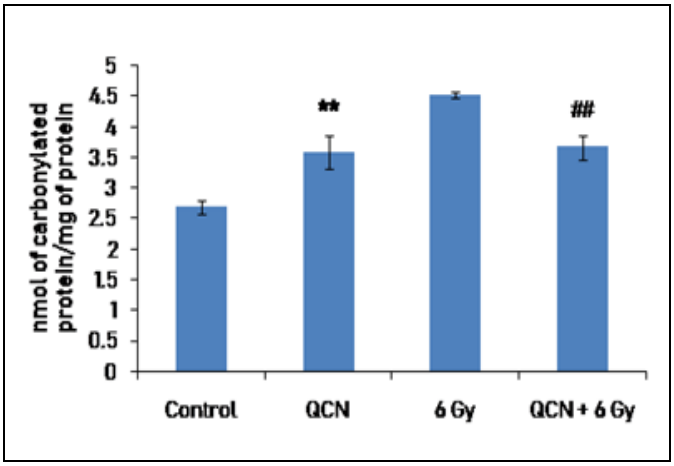

FIG. 2: QCN HAS A PRO-OXIDANT ACTION. BAR GRAPH SHOWING NMOL OF CARBONYLATED PROTEIN PER MG OF PROTEIN AFTER VARIOUS TREATMENT. **p< 0.01 *vs.* control and ##p<0.01 *vs.* 6 gy group. Values are expressed in terms of mean ± standard deviation

QCN has a Pro-oxidant Action: We checked the levels of carbonylated proteins as it serves as an indicator of protein damage due to free radicals [24] **Fig. 2**. We observed that QCN when used alone, had a pro-oxidant action (**p<0.01); however, QCN lowered the free radicals in the combination group as compared to the 6 Gy radiation alone group (##p<0.01).

QCN Lowers Levels of Pro-metastatic Proteins: We performed western blotting to check the levels of the pro-metastatic proteins MMP-2 **Fig. 3A** and **3B** and p21 **Fig. 3A** and **3C** in response to the different treatment conditions. QCN, when used alone, lowered the levels of both MMP-2 and p21 as compared to the control (**p<0.01; in case of MMP-2 and *p<0.05; in case of p21). In case of the

combination group, the levels of both MMP-2 and p21 were significantly lowered as compared to the 6 Gy radiation alone group (##p<0.01 in both cases).

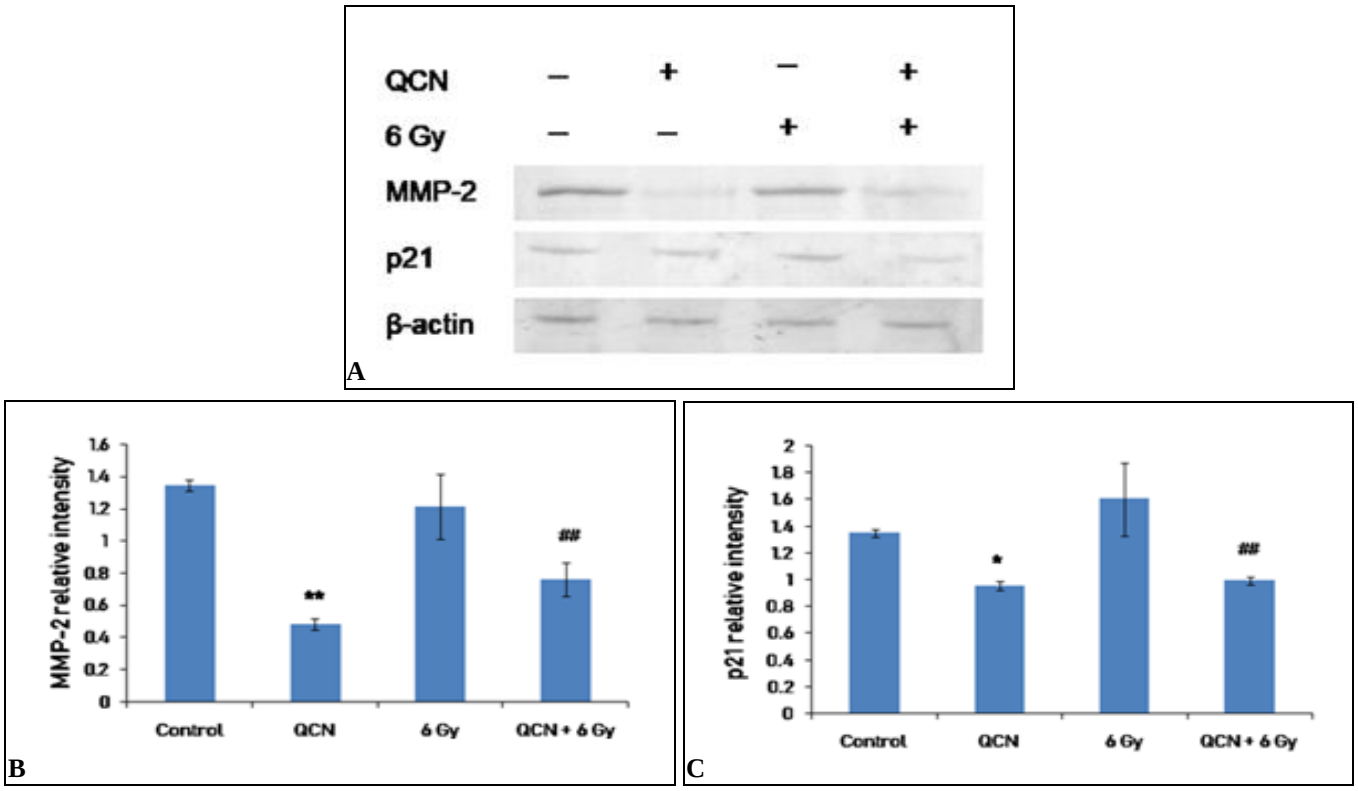

FIG. 3: QCN REDUCES THE PROTEIN LEVELS OF MMP-2 AND P21. (A) WESTERN BLOTTING IMAGES OF MMP-2 AND P21 PROTEINS. DENSITOMETRIC PLOTS OF THE LEVEL OF (B) MMP-2 PROTEIN, **P< 0.01 *vs.* CONTROL AND ##P< 0.01 *vs.* 6 GY GROUP. (C) P21 PROTEIN, *P< 0.05 *vs.* CONTROL AND ##P< 0.01 *vs.* 6 GY GROUP. All groups were normalized by taking β-actin as a reference protein.

QCN Up-regulates PTEN Expression and Down-regulates AKT1 Expression: QCN promotes the gene expression levels of the PTEN gene **Fig. 4A** when it was used alone as compared to the control (**p<0.01). The PTEN expression levels registered a sharp increase in the 6 Gy radiation group while the PTEN expression level was again lowered in the combination group (##p<0.01). In case of AKT1 expression levels **Fig. 4B**, there was a down-regulation in the AKT1 mRNA levels in both the QCN treated groups (**p<0.01 *vs.* control and ##p<0.01 *vs.* 6 Gy group; respectively).

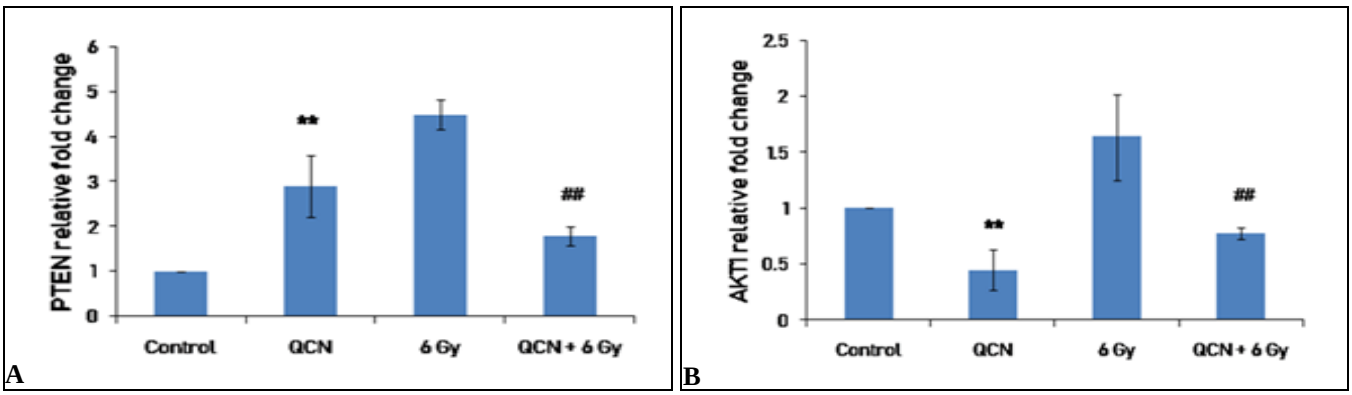

FIG. 4: QCN UP-REGULATES PTEN AND DOWN-REGULATES AKT1. FOLD CHANGE IN MRNA EXPRESSION OF (A) PTEN, **P< 0.01 *vs.* CONTROL AND ##P< 0.01 *vs.* 6 GY GROUP; AND (B) AKT1. **P< 0.01 *VS.* CONTROL AND ##P< 0.01 *vs.* RESPECTIVE IRRADIATED GROUP. All values were normalized with ACTB endogenous control. All values are expressed as Mean ± standard deviation.

QCN Promotes BAX and BECN1: QCN caused a lowering in the levels of the BCL2 mRNA when the H23 cells were treated with QCN alone (**p<0.01) **Fig. 5A**. The radiation-treated groups didn't show any significant changes in the level of BCL2 gene expression. BCL-XL mRNA levels were lowered **Fig. 5B** both when QCN was used alone and in combination with radiation (**p<0.01 and #p<0.05; respectively). BAX gene expression levels were elevated **Fig. 5C** in the H23 cells both when QCN was used alone (**p<0.01) and in combination with 6 Gy radiation (##p<0.01). There was an up-regulation of the BECN1 gene expression levels **Fig. 5D** in the QCN treated groups, with the combination group registering the highest increase in the BECN1 gene expression levels (**p<0.01 and ##p<0.01; respectively). CASPASE3 gene expression levels showed an approximately two-fold increase in the cancer cells when the cells were treated with QCN alone (**p<0.01). The 6 Gy group registered a six-fold increase in CASPASE3 gene expression amongst all the treated groups, while the expression levels fell down to approximately two-fold of the untreated control in the combination group (#p<0.01).

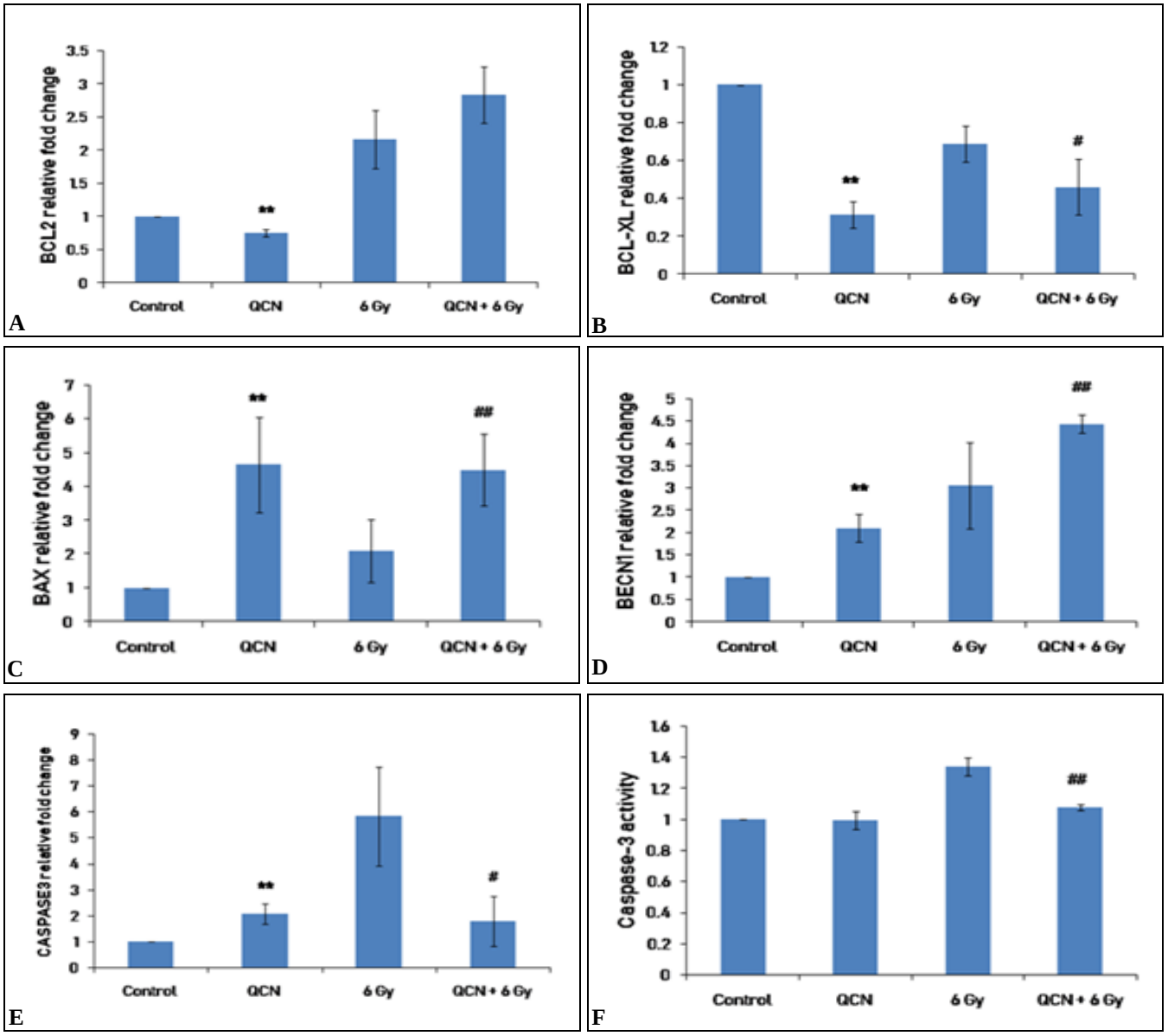

FIG. 5: QCN ELEVATES BAX AND BECN1. FOLD CHANGE IN THE MRNA EXPRESSION OF (A) BCL2, **P< 0.01 *vs.* CONTROL; (B) BCL-XL, **P<0.01 *vs.* CONTROL AND #P< 0.05 VS. 6 GY GROUP; (C) BAX, **P< 0.05 *vs.* CONTROL AND ##P< 0.01 *vs.* 6 GY GROUP; (D) BECN1, **P<0.01 *vs.* CONTROL AND ##P<0.01 *vs.* 6 GY GROUP; (E) CASPASE3, *P< 0.05 *vs.* CONTROL. ALL VALUES WERE NORMALIZED WITH ACTB ENDOGENOUS CONTROL; (F) GRAPH FOR FOLD CHANGE IN CASPASE-3 ACTIVITY, ##P< 0.01 *vs.* 6 GY GROUP. All values are expressed as mean ± standard deviation.

The caspase-3 activity stayed unchanged in the QCN alone group with respect to the control group. The combination group showed a decreased activity of caspase-3 as compared to the 6 Gy group (##p<0.01).

DISCUSSION: With the cytotoxic effect of QCN on H23 cells previously established [27], we moved into checking the ability of QCN to radiosensitize the H23 cells. From the results of the cell proliferation assay **Fig. 1A** and **Table 1** we observed that QCN in combination with radiation could lower the cell survival of the H23 cells as compared to the radiation alone treated groups. The lowering of cell survival in the combination groups of QCN and radiation was specific to the cancer cell line H23 as the same combination of treatment didn't cause any lowering in the number of viable embryonic HEK293T cells **Fig. 1B** and **Table 2**.

Another important aspect is that QCN achieved an approximately 50% lowering in cancer cell survival in combination with 6 Gy while in case of the embryonic HEK293T cells the same combination of QCN + 6 Gy rather produced a higher count of viable cells as compared to the 6 Gy alone group. Although QCN in combination with 8 Gy radiation produced a lowering of H23 cell survival by almost 37% but on the flip side at that combination, the HEK293T cells registered a more than 50% decrease in the number of viable cells **Table 1** and **2**. Since, QCN achieved the dual purpose of causing a 50% lowering of cancer cell survival while also being safe for the embryonic cells when QCN was used in combination with 6 Gy radiation; we decided to move ahead with the rest of the study with a single dose of 6 Gy radiation.

We checked for viable H23 cells using membrane integrity as a marker of viability 22 and observed that QCN when used alone and in combination with 6 Gy radiation, caused a significant lowering of viable cells **Fig, 1C** and also caused an approximately four-fold increase in dead cells **Fig. 1D**. The inherent sources of error in the cell proliferation assay and cell viability assays could be that cancer cells could slow down their metabolism or go into a cell cycle arrest in response to treatment or a segment of the cancer cells might show delayed membrane breakage [28]. The former situation could affect the results of the MTT based cell proliferation assay, while the latter situation would invariably affect the results of the cell viability assay. Thus to ensure that our results were not influenced by the above factors, we performed the colony-forming assay **Fig. 1E** and **1F**. The calculated percent cell survival pattern from the colony-forming assay mirrored the results we attained from cell proliferation and cell viability assays with both the QCN treated groups showing lowered cell surviving thereby further confirming the efficacy of QCN as a potential radiosensitizer of lung adenocarcinoma cells.

From the protein carbonylation measurements, we could decipher that QCN, when used alone and in combination with 6 Gy radiation, with respect to the untreated control cells, caused an increase in the reactive oxygen species and a subsequent increase in oxidative stress leading to the increased amount of carbonylated proteins. The increase in oxidative stress in the QCN treated groups can be attributed to the free radical scavenging activity of QCN, which ends up converting QCN to QCN radicals and also to the glutathione (GSH) depleting and glutathione S transferase lowering activity of QCN, which ultimately results in increased oxidative stress and also leads to cell death [29].

QCN, when used singularly and in combination with radiation, was able to lower the protein levels of the pro-metastatic proteins MMP-2 **Fig. 3A** and **3B** and p21 **Fig. 3A** and **3C**. The lowering of MMP-2 not only lowers the metastatic potential but also inhibits the transforming growth factor beta (TGF-β) dependent pathways for EMT 12 and activation of cell proliferation promoting pathways like the Akt pathway [30]. p21 lowering allowed the lowering of p21 associated EMT and cell proliferation [11].

Both MMP-2 and p21 are associated with increased resistance of lung adenocarcinoma cells towards therapeutic procedures like radiation [9, 17] and the lowering of both these proteins in the QCN treated groups were associated with the lower H23 cell survival thus showing QCN in combination with radiation could act a radiosensitizer *via* lowering MMP-2 and p21 proteins. QCN; when used alone and in combination with 6 Gy radiation; elevated the expression of the tumour suppressor PTEN

gene **Fig. 4A** while lowering the expression of the pro-survival AKT1 gene **Fig. 4B**. QCN has been previously reported to elevate PTEN expression *via* peroxisome proliferator-activated receptor gamma (PPARγ) dependent pathway in different cancer cell lines [31]. Elevation of PTEN allows the observed lowering of AKT1 gene as PTEN prevents the accumulation of β-catenin in the nucleus which is needed for AKT1 expression [32, 33]. PTEN activates the forkhead box O3 (FoxO3a) transcription factor, which is involved in promoting the expression of apoptotic and autophagic proteins [34]. AKT1 is involved in inhibiting the activity of FoxO3a transcription factor, thereby promoting cell survival [35].

AKT1 is also involved in promoting the activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) [36,] which in turn promotes the expression of pro-survival, anti-apoptotic, and pro-metastatic proteins; including both MMP-2 and p21 [37, 38]. Thus in the QCN treated samples with elevated PTEN expression and lowered AKT1 expression, we observed a reduction in the levels of MMP-2 and p21 along with a reduction in the percent cell survival. PTEN expression is promoted, but its activity is inhibited under high oxidative stress [39] while AKT1 expression and activity is promoted via increased Src proto-oncogene (Src) activity in high oxidative stress [40]. Thus in the 6 Gy group, there was a high level of carbonylated proteins and high PTEN and AKT1 expression but a minimal reduction in cell survival as compared to the untreated control.

QCN, when used singularly, caused a down-regulation of the anti-apoptotic BCL2 expression however, there was no change in the BCL2 mRNA levels in the radiation-treated groups **Fig. 5A**. The levels of anti-apoptotic BCL-XL mRNA were lowered both when QCN was used singularly and in combination with radiation **Fig. 5B**.

In the case of lung adenocarcinomas, BCL-XL plays a more prominent role in cancer progression than BCL2 as BCL-XL is involved in the promotion of metastasis [41], inhibition of apoptosis [41,] and autophagy [42,] and promotion of the Akt pathway [41]. Thus the lowering of BCL-XL in the QCN treated groups has multifaceted advantages. QCN treated groups showed an increased expression of the pro-apoptotic BAX mRNA **Fig. 5C**. Increase in BAX is significant as BAX can promote both caspase dependent and caspase independent apoptosis [43, 44]. QCN treated groups also caused an increase in the expression of autophagy gene BECN1 with the combination group showing an approximately four-fold increase in BECN1 expression **Fig. 5D**. BECN1 can promote both autophagy and apoptosis46 and also inhibit metastasis [45].

Thus BECN1 can be considered as a contributing factor to the earlier observed results. In case of CASPASE3 gene expression **Fig. 5E**, there was a modest increase in mRNA levels of CASPASE3 in the QCN treated groups while the caspase-3 activity **Fig. 5F** showed no significant changes in the activity levels in the QCN treated groups as compared to the control. Caspase-independent apoptosis associated with QCN has been reported in other cell lines [46].

The lack of caspase-3 activity in the QCN treated groups show that QCN induced cell death *via* caspase-3 independent mechanisms, thereby further implicating the role of oxidative stress, BAX, and BECN1 in the contribution towards cell death.

CONCLUSION: Based on our results, we can summarize by stating that QCN had the ability to sensitize the lung adenocarcinoma cell line H23 to radiation. QCN was able to achieve radio-sensitization *via* up-regulation of PTEN gene, down-regulation of AKT1 gene, lowering of the metastatic proteins MMP-2 and p21, and by promoting oxidative stress. QCN promoted a BAX, BECN1 and oxidative stress-dependent and a caspase-3 independent manner of cell death in the H23 cells.

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CONFLICTS OF INTEREST: None to declare.

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