Original Article

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1,4-Naphthoquinone, a pro-oxidant, ameliorated radiation induced gastro-intestinal injury through perturbation of cellular redox and activation of Nrf2 pathway

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Summary **Detrimental effects of ionizing radiation (IR) are observed at the doses above 1 Gy. Treatment modalities are available up to doses of 6 Gy including bonemarrow transplantation and administration of antibiotics. However, exposure to IR doses above 8 Gy results in gastro-intestinal (GI) syndrome characterised by denudated villi, apoptosis of crypt cells and elevated inflammatory responses. Multiple strategies have been employed to investigate novel agents to protect against IR induced injury. Since cellular redox homeostasis plays a pivotal role in deciding the cell fate, present study was undertaken to explore the potential of 1,4-naphthoquinone (NQ), a pro-oxidant, to ameliorate IR induced GI syndrome. NQ protected INT 407 cells against IR induced cell death of intestinal epithelial cells *in vitro*. NQ induced perturbation in cellular redox status and induced the activation of nuclear factor-erythroid 2-related factor 2 (Nrf2) pathway. Thiol antioxidant and inhibitors of Nrf2 pathway abrogated the radioprotection offered by NQ. Further, knocking down Nrf2 rescind the NQ mediated protection against IR induced cell death. In conclusion, NQ protects against IR radiation induced GI syndrome *in vitro* by perturbing cellular redox and activating Nrf2 pathway. This is the first report highlighting the potential of a pro-oxidant to ameliorate IR induced GI injury.**

Keywords: Radioprotection, cellular redox status, ERK/Nrf2 pathway, Nrf2 knockdown

1. Introduction

Increased use of ionising radiation (IR) as treatment modality in multiple carcinomas and augmented risk of unplanned exposure due to nuclear proliferation and radioactive waste disposal have potentiated the need of a potent radioprotective agent which would be useful in multiple operational scenarios. Acute illness caused due to high doses of IR is termed as acute radiation syndrome (ARS). ARS is a dose dependent damage affecting the hematopoietic, gastro-intestinal (GI), cerebrovascular and cutaneous systems in the body (*1,2*). Rapidly proliferating cells and their respective organs are more sensitive towards IR induced cell death. Epithelial cells located in the small intestinal crypts divide rapidly and are among the most susceptible cells in the body to IR induced death. Higher susceptibility of GI tract to IR is the major limiting factor in radiation therapy of abdominal and pelvic malignancies (*3-5*).

Acute effects after doses above 8 Gy are known as gastrointestinal syndrome which causes mortality in 8 to 12 days. GI syndrome is characterized by loss in the absorptive surface of small intestine called as jejunum due to denudation of villi and apoptosis in submucosal invaginations called crypts of Lieberkuhn resulting in vomiting, diarrhoea, anorexia, dehydration, systemic infection and septic shock induced death in severe cases (*6*). Each crypt in jejunum contains intestinal stem cells (ISC) at the base which are responsible to supply the differentiated enterocytes to maintain homeostasis (*7,8*). Lineage-tracing experiments illustrated that a $Lgr5^+$ cell produced terminally differentiated epithelial lineages of mouse intestine (*9*). Further, a single $Lgr5^+$ stem cell is capable of generating crypt/villus organoids i*n vitro*

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containing differentiated intestinal mucosa cell lineages (*10*). However, exposure to radiation induces apoptosis in ISC, thus delimiting their potential to repopulate the denudated absorptive surface (*4,8,11*).

Since large part of IR induced damages are mediated due to generation of reactive oxygen species (ROS), much of the efforts in past were targeted to explore the potential of antioxidants to ameliorate IR induced toxicities. However recent advances have highlighted that activation of pro-survival pathways could serve as a novel target in developing a potent radioprotector. CBLB502 protected against IR induced mortality due to GI syndrome by activating NF-κB pathway (*12*). An anti-ceramide monoclonal antibody protected against apoptosis in the small intestine and whole body irradiation (WBI) induced mortility in mice (*13*). Lactobacillus probiotic was shown to protect against WBI induced apoptosis in intestinal epithelial cells in TLR-2 and cyclo-oxygenase-2 dependent manner (*14*). Sphingosine-1-phosphate, a ceramide antagonist, protected against WBI induced endothelial apoptosis in intestine by activating Akt pathway (*15*). R-spondin 1 improved survival percentage in mice exposed to WBI doses inducing GI syndrome by activating Wnt/β-catenin pathway (*16*). Despite of the potent efficacy of investigated agents, associated toxicity delimits their use in operational scenarios creating the dearth of agents with prolonged efficacy, broad specificity, and minimal toxicity to increase the benefit of radiation therapy in cancer treatment *(2*).

Recently, activation of redox sensitive pro-survival transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2) was shown to protect colonic epithelial cells from IR induced loss in cell viability (*17*). Activation of Nrf2 was shown to protect against oxidative stress induced cell death implicated in pathogenesis of ischemia, inflammation and cancer. Nrf2 has been shown to control the expression of intestinal detoxification and intestinal stem cell proliferation in drosophila. Previously, 1,4-naphthoquinone (NQ), a pro-oxidant, was shown to ameliorate radiation induced hematopoietic syndrome and exhibit anti-inflammatory effects by activation of Nrf2 pathway (*18,19*). Based on the embroiled role of Nrf2 in maintaining intestinal homeostasis, we hypothesised that modulation of cellular redox mediated activation of Nrf2 by a pro-oxidant might provide an adaptive response to confer protection against radiation induced GI syndrome. In the present study we highlight the potential of NQ, a pro-oxidant, to ameliorate radiation induced GI syndrome by activating Nrf2 pathway.

2. Materials and Methods

2.1. *Chemicals*

NQ, HEPES, ethylenediaminetetracetate (EDTA), ethylene glycol tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), dimethyl sulfoxide (DMSO), leupeptin, aprotinin, benzamidine, dithiothreitol (DTT), dichlorofluorescein diacetate (H2DCF-DA), penicillin, streptomycin, crystal violet, N-acetyl cysteine (NAC) and Nonidet P-40 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). cDNA synthesis kit, chemiluminiscence kit and SYBR green PCR mix were procured from Roche Chemical Co. (San Francisco, CA, USA). Roswell Park Memorial Institute (RPMI-1640) medium and fetal bovine serum (FBS) was obtained from HiMedia (Kennett Square, PA, USA). All-trans-retinoic acid (ATRA), PD98059 (ERKi) and JC-1 fluorescent dye were purchased from Calbiochem (Billerica, Massachusetts, USA). Hoechst 33342 was procured from Invitrogen (Carlsbad, CA, USA). Antibodies against pERK, ERK, pMEK, MEK and β-actin were procured from Cell Signalling Technologies (Danver, MA, USA). Anti-Nrf2 antibody and oligonucleotide probe for Nrf2 was procured from Santacruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were purchased from reputed local manufacturers.

2.2. *Cell culture*

INT 407 (human intestinal epithelial cell line) cells were obtained from Health Protection Agency Culture Collections (HPACC, Salisbury, Wiltshire, UK) were cultured in Roswell Park Memorial Institute (RPMI-1640) medium containing 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) at 37°C in an atmosphere of 5% CO_2.

2.3. *Treatment with NQ*

Stock solution of NQ was prepared in DMSO, stored as small aliquots at -20ºC. In all *in vitro* experiments, cells were treated with different doses of NQ for 2 h or NQ 1 µM for indicated time interval before the initiation of culture. DMSO was used as vehicle control.

2.4. *Irradiation schedule*

INT 407 cells in medium were irradiated at a dose rate of 1.2 Gy/min in a blood irradiator (BRIT, Mumbai, India).

2.5. *Measurement of change in mitochondrial membrane potential (MMP)*

INT 407 Cells were treated with NQ 1 µM for 2 h followed by radiation dose of 15 Gy and were cultured for 24 h at 37°C. MMP was assessed using the mitochondrial-specific cationic fluorescent probe JC-1 (10 µM) by spectroflourimetric method as described previously (*20*).

2.6. *Caspase 3 assay*

INT 407 Cells were treated with NQ 1 μM for 2 h followed by radiation dose of 15 Gy and were cultured for 24 h at 37°C. Caspase 3 activity was measured following manufacturer`s protocol (Caspase 3 Assay Kit, Colorimetric, Sigma Aldrich, St. Louis, MO, USA).

2.7. *Clonogenic cell survival assay*

One thousand INT 407 cells were cultured in 100-mm culture dishes and treated with ATRA (Nrf2 inhibitor) or PD98059 (ERK inhibitor) or NAC for 2 h followed by treatment with NQ 1 μM for 2 h and then irradiated with dose of 6 Gy or 7 Gy or 8 Gy. Cells were allowed to grow for 14 days to form colonies, which were then stained with crystal violet (0.4 g/L), and colonies were counted using colony counter (*20*).

2.8. *Intracellular ROS measurements*

INT 407 cells were incubated with 20 μM oxidation-sensitive dichlorofluorescein diacetate (H_2DCF-DA) for 25 min at 37°C and then treated with NQ 1 μM for indicated time points. After incubation, the increase in fluorescence resulting from oxidation of H2DCF to DCF was measured using a spectrofluorimeter (*21*).

2.9. *Determination of intracellular glutathione (GSH) and glutathione disulfide (GSSG) levels*

INT 407 cells were pre-treated with NQ 1 μM for indicated points. Intracellular GSH/GSSG ratio was measured spectrophotometrically by conventional enzyme cycling method (*22*).

2.10. *Quantitative Real Time PCR*

INT 407 cells were pre-treated with NQ 1 μM for indicated points. RNA isolation, cDNA synthesis and RT-PCR were performed as described previously (*21*). Briefly, total RNA was isolated from the samples using Trizol reagent. One μg of total RNA was used for preparation of cDNA by reverse transcription (cDNA synthesis kit, Roche Chemical Co, San Francisco, CA, USA) following the manufacturer's instructions. Quantitative PCR was carried out using a Rotor Gene 3000 (Corbett Research, Mortlake, NSW, Australia) machine. The threshold cycle values were used for calculating the expression levels of genes by REST-384 version-2 software (*23*). PCR efficiency was calculated for individual primer pairs. Specific primer sequences were obtained from primer bank (Table 1). Expression of the genes was normalized against a house keeping gene, β-actin and plotted as relative change in expression with respect to control.

Table 1. List of specific primers for RT-PCR

Gene	Sequence
HO-1	Forward: AGGTACACATCCAAGCCGAGA
	Reverse: CCATCACCAGCTTAAAGCCTT
GCLC	Forward: CTACCACGCAGTCAAGGACC
	Reverse: CCTCCATTCAGTAACAACTGGAC
Nrf2	Forward: CTTTAGTCAGCGACAGAAGGAC
	Reverse: AGGCATCTTGTTTGGGAATGTG
MnSOD	Forward: CAGACCTGCCTTACGACTATGG
	Reverse: CTCGGTGGCGTTGAGATTGTT
Catalase	Forward: AGCGACCAGATGAAGCAGTG
	Reverse: AGGACATCAGGTCTCTGCGA
β-actin	Forward: GCGGGAAATCGTGCGTGACATT
	Reverse: GATGGAGTTGAAGGTAGTTTCGTG

2.11. *Western blot analysis*

INT 407 cells were pre-treated with different concentrations of NQ for 12 h or NQ (1 μM) for indicated time points. Cytosolic extracts were prepared as described earlier (*20*). Vehicle treated cells served as a control. Equal amount of protein was resolved by SDS-PAGE (10%), transferred to nitrocellulose membrane, blocked and incubated overnight with the primary antibody specific to pERK, ERK, pMEK, MEK and β-actin. After subsequent washing, membrane was further incubated with horseradish peroxidase-labeled secondary antibody for 2 h and specific bands were visualized on X-ray films using Enhanced Chemiluminiscence Kit. β-actin was used as loading control.

2.12. *Determination of nuclear levels of Nrf2 using confocal microscopy*

INT 407 cells were treated with NQ (1 μM) at indicated time intervals. The cells were labelled with anti-Nrf2 antibody as described previously (*24*). Further, these cells were stained with FITC-labelled secondary antibody followed by PI staining. Slides were examined using an LSM510 confocal microscope (Carl Zeiss, Jena, Germany) with a krypton-argon laser coupled to an Orthoplan Zeiss photomicroscope.

2.13. *Electrophoretic mobility shift assay*

INT 407 cells were pre-treated with NQ 1 μM for indicated time points and different concentrations of NQ for 12 h at 37°C. Nuclear extracts were prepared as described earlier (*19*). EMSA was performed by incubating 8μg of nuclear proteins with 16 fmol of ^{32}P-end-labeled, 45-mer double stranded NF-κB oligonucleotides from the human immunodeficiency virus long terminal repeat (5′-TTGTTACAA**GGGA CTTTCCGCTGGGGACTTTCC**AGGGAGGCG TGG-3′; boldface indicates NF-κB binding sites) in the presence of 0.5 μg of poly (2′-deoxyinosinic-2′-deoxycytidylic acid) in binding buffer for 30 min

at 37°C. The DNA-protein complex formed was separated from free oligonucleotide on 7.6% native polyacrylamide gels. The dried gel was exposed to phosphorimage plate and the radioactive bands were visualized using a PhosphorImage plate scanner (Amersham Biosciences, Piscataway, NJ, USA).

2.14. *shRNA knockdown studies*

Nrf2 shRNA plasmid (cat no. TF515053, Origene, Rockville, MD, USA) was used for knocking down Nrf2 in INT 407 cells. Transfection was performed using Neon® Transfection System (Invitrogen, Carlsbad, CA, USA) following manufacturer`s protocol. Briefly, 5 million cells were electroporated (pulse voltage 1005 and pulse width 35 ms) using 1 µg of nrf2 shRNA in antibiotic free medium. Cells were further cultured for 48 h for transgene expression. Knockdown INT 407 cells were treated with NQ 1 µM for 2 h followed by radiation dose of 6 Gy. Cells were further subjected to clonogenic assay as discussed earlier (*20*).

2.15. *Statistical analysis*

The statistical analysis was done using analysis of variance with Microcal OriginPro 8.0 software followed by post hoc analysis using Schiffe's test. * refers to $p < 0.01$, as compared to control, # refers to $p < 0.01$ as compared to irradiated group and $ refers to $p < 0.01$ as compared to NQ + IR group. Data points represent mean ± SEM from three replicates, and two independent experiments were performed.

3. Results

3.1. *NQ significantly protected against radiation induced cell death of INT 407 cells*

INT 407, a human intestinal epithelial cell line, was used as an *in vitro* model system to decipher the potential of NQ to protect against radiation induced GI syndrome. Cells were pre-treated with NQ 1 µM for 2 h followed by exposure to radiation 6 Gy or 7 Gy or 8 Gy. Vehicle treated cells served as control. Effect of radiation on viability of INT 407 cells was assessed by monitoring changes in proliferating potential which is defined by the ability of a single cell to grow as a colony. Clonogenic assay is the method of choice to determine cell reproductive death after treatment with radiation. Exposure to radiation induced significant reduction in the percent surviving fraction. Interestingly, treatment with NQ significantly (55% at 6 Gy, 38% at 7 Gy and 28% at 8 Gy) protected against radiation induced loss of clonogenicity (Figure 1A). Further, modulation in mitochondrial membrane potential and caspase 3 activity was also monitored to decipher the effect of NQ on radiation induced apoptosis. Vehicle treated cells served as control. Exposure to radiation induced a significant reduction in MMP (64%) and increase in caspase 3 activity indicating the induction of

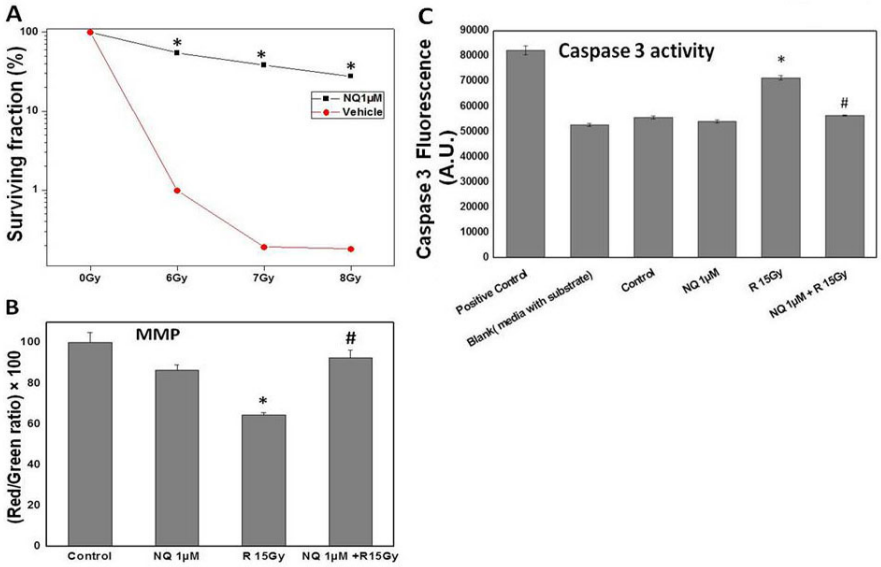

Figure 1. NQ offered radioprotection in INT 407 cells. (A) INT 407 cells were treated with NQ 1 µM for 2 h followed by radiation exposure of 6 Gy or 7 Gy or 8 Gy. Vehicle treated cells served as control. Cells were incubated for 14 days to monitor the clonogenic potential. Line graphed is the percent surviving fraction at the indicated doses of IR. **(B)** Cells were treated with NQ 1 µM or vehicle for 2 h followed by exposure to radiation dose of 15 Gy and were incubated for 24 h. Cells were harvested and stained with JC-1 dye to monitor IR induced loss in mitochondrial membrane potential in respective groups. Graphed is the percentage loss of mean (red/green) fluorescence of JC-1. **(C)** NQ inhibited IR induced increase in Caspase 3 activity. Cells were treated with NQ 1 µM or vehicle for 2 h followed by exposure to radiation dose of 15 Gy and were incubated for 24 h. Graphed is the arbitrary fluorescence unit representing the caspase 3 activity. Each bar represents mean ± S.E.M. from three replicates and two such independent experiments were carried out. The statistical analysis was done using analysis of variance followed by post hoc analysis using Schiffe's test. *$p < 0.01$, as compared to control, # refers to $p < 0.01$ as compared to irradiated group.

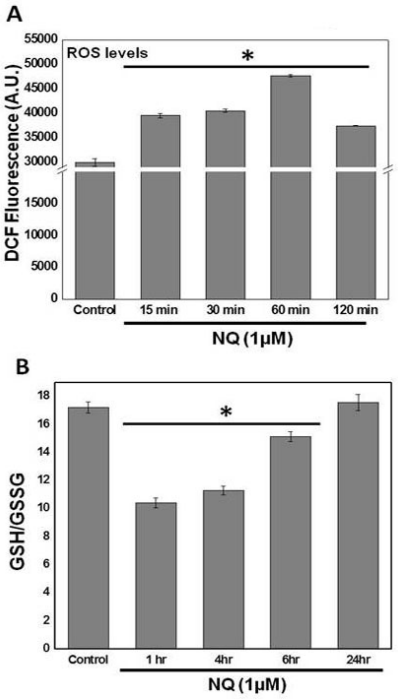

Figure 2. NQ induced perturbation of cellular redox status. (A) NQ induced a transient increase in the basal ROS levels. DCFH2-DA-stained INT 407 were treated with NQ 1 μM for indicating time intervals at 37°C and fluorescence emission was measured at 535 nm. **(B)** NQ induced a transient decrease in the levels of redox couple GSH/GSSG. INT 407 cells were treated with NQ 1 μM for indicated time intervals. GSH/GSSG ratio was estimated using enzyme cycling method. Each bar represents mean ± S.E.M. from three replicates and two such independent experiments were carried out. The statistical analysis was done using analysis of variance followed by post hoc analysis using Schiffe's test. *$p < 0.01$, as compared to control.

apoptosis. Interestingly, treatment with NQ significantly ameliorated radiation induced apoptosis by protecting against loss of mitochondrial membrane potential (92%) and abrogated the increase in caspase 3 activity (Figures 1B and 1C).

3.2. *NQ modulated cellular redox status in INT 407 cells*

Cellular redox status is impervious for normal functioning of cell. Pro-oxidants being a strong electrophile are known to modulate the cellular redox status which serves as the prime mediator of its effect. Effect of NQ to modulate cellular redox status was evaluated by monitoring changes in ROS and GSH/GSSG levels. DCFDA stained INT 407 cells were treated with NQ 1 μM at indicated time points to monitor the changes in levels of ROS. NQ treatment induced a transient increase in basal ROS levels (Figure 2A) indicating its pro-oxidant effect. GSH/GSSG, endogenous antioxidant, is a major redox couple involved in maintaining the cellular redox homeostasis. Being a strong electrophile, NQ exhibits a strong attraction towards cellular nucleophiles like GSH and may modulate their levels. Thus, effect of NQ was observed on the levels of GSH/GSSG. NQ induced a fleeting depletion in GSH/GSSG ratio which with long time exposure came back to control levels (Figure 2B). These results further highlight the non toxic effect of NQ as incessant depletion in the levels of GSH may be associated to cell death.

3.3. *NQ induced activation of Nrf2, a pro-survival transcription factor, pathway in INT 407 cells*

NQ previously has been shown to activate mitogen activated protein kinases (MAPK) in lymphocytes (*18*). Also, perturbation in cellular redox status is known to activate key redox sensitive regulatory transcription factor Nrf2. Therefore, potential of NQ to activate MAPK upstream of Nrf2 was explored. MEK-1 is a dual threonine and tyrosine recognition kinase that phosphorylates and activates extracellular-signal-regulated kinases (ERK). NQ induced activation of MEK and ERK, upstream kinases in Nrf2 pathway, in concentration and time dependent manner (Figure 3A). Since NQ modulated cellular redox status and activated upstream ERK, the effect of NQ on redox sensitive cytoprotective transcription factor Nrf2 was monitored. NQ increased the mRNA levels of Nrf2 and its dependent cytoprotective/antioxidant genes (MnSOD, HO-1, GCLC and Catalase) in a time dependent manner (Figure 3B). INT 407 cells were treated with NQ 1 μM for different time points to monitor the nuclear translocation of Nrf2. NQ induced Nrf2 nuclear translocation at 12 h and 24 h (Figure 3C). Further, INT 407 cells were treated with different concentrations of NQ for 12 h to monitor the binding of Nrf2 to its consensus sequence (Antioxidant Response Element). It was observed that NQ markedly increase the activation of Nrf2 as evident by enhanced DNA binding in EMSA (Figure 3D).

3.4. *Thiol antioxidants and Nrf2 inhibitor abrogated NQ mediated protection against radiation induced cell death*

Contribution of NQ induced perturbation of cellular redox status and activation of ERK/Nrf2 pathway in its radioprotective effect was monitored by employing pharmacological inhibitors ERKi (PD98059, ERK inhibitor) and ATRA (Nrf2 inhibitor) and NAC (thiol antioxidant). Clonogenic method was employed to monitor the effect of inhibitors on radioprotective potential of NQ. Employing these inhibitors alone does not offer any protection against radiation induced loss of proliferating potential in clonogenic assay. NQ treatment significantly (41% surviving fraction) protected against radiation induced loss (5% surviving fraction) of clonogenicity. Intriguingly, employment of NAC and ERKi along with NQ showed 11% and 15% surviving fraction indicating a significant abrogation of the NQ mediated radioprotection. Employing

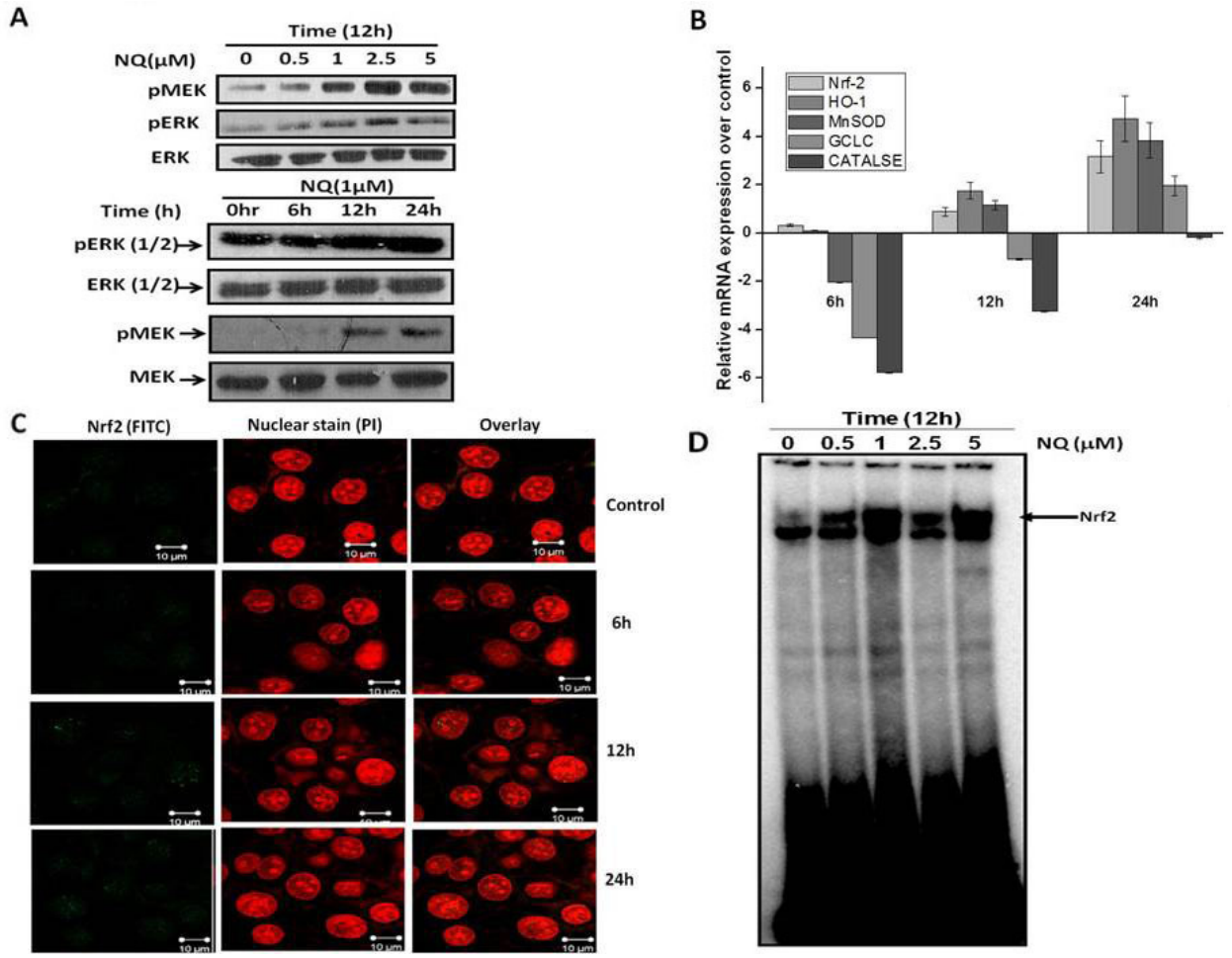

Figure 3. NQ induced activation of ERK/Nrf2 pathway. (A) INT 407 cells were treated with different concentrations of NQ for 12 h or with NQ 1 μM for indicated time intervals. Cytoplasmic extracts were fractionated on 10% non-reducing SDS-PAGE, and electrotransferred to nitrocellulose membrane. Western blot analysis was performed using antibodies specific for pMEK, MEK, pERK, ERK and β-actin. **(B)** INT 407 cells were treated with NQ 1 μM for 6 h, 12 h and 24 h, mRNA was isolated and used for real time RT-PCR. Bar diagram shows relative mRNA copy number of Nrf2, HO-1, MnSOD, GCLC and Catalase over control. Each bar represents mean ± S.E.M. from three replicates and two such independent experiments were carried out. **(C)** INT 407 cells were treated with NQ 1 μM for 6 h, 12 h and 24 h, stained with FITC labelled anti-Nrf2 antibody and PI. FITC (left)/PI(mid)/ overlay(right) is shown. **(D)** INT 407 cells were treated with NQ 1 μM for 6 h, 12 h and 24 h, EMSA was performed using nuclear extracts.

ATRA showed complete abrogation (4% surviving fraction equivalent to radiation treatment) of NQ induced protection against radiation induced loss of clonogenicity in INT 407 cells (Figures 4A and 4B). These results indicated that the modulation in cellular redox and activation of Nrf2 might be the underlying mechanism of NQ mediated protection against radiation induced GI syndrome.

3.5. *Knocking down Nrf2 significantly abrogated the NQ mediated radioprotection*

Knock down studies were adopted to contemplate the central role of Nrf2 pathway in NQ mediated protection against radiation induced cell death. INT 407 cells were transfected with Nrf2 shRNA or scrambled shRNA to create a transient knock down. After 24 h, transfected cells with ablated Nrf2 were washed and treated with NQ 1 μM for 2 h prior to radiation exposure of 6 Gy and were further monitored for loss in clonogenicity. NQ treatment offered significant (57% survival fraction) protection against radiation induced cell death in mock control and scrambled shRNA transfected cells. Interestingly, knocking down Nrf2 pathway completely abrogated (8% survival fraction in cells with ablated Nrf2) the NQ mediated protection against radiation induced clonogenic death (Figures 5A and 5B). Further, ablation in ERK and Nrf2 dependent cytoprotective gene, HO-1, also showed the significant abrogation of NQ mediated radioprotection (data not shown). These results contemplated our hypothesis that activation of Nrf2 is a central regulator and pivotal for NQ induced protection against radiation induce GI syndrome.

4. Discussion

Maintenance of cellular redox homeostasis is impervious to maintain cell viability and normal physiological

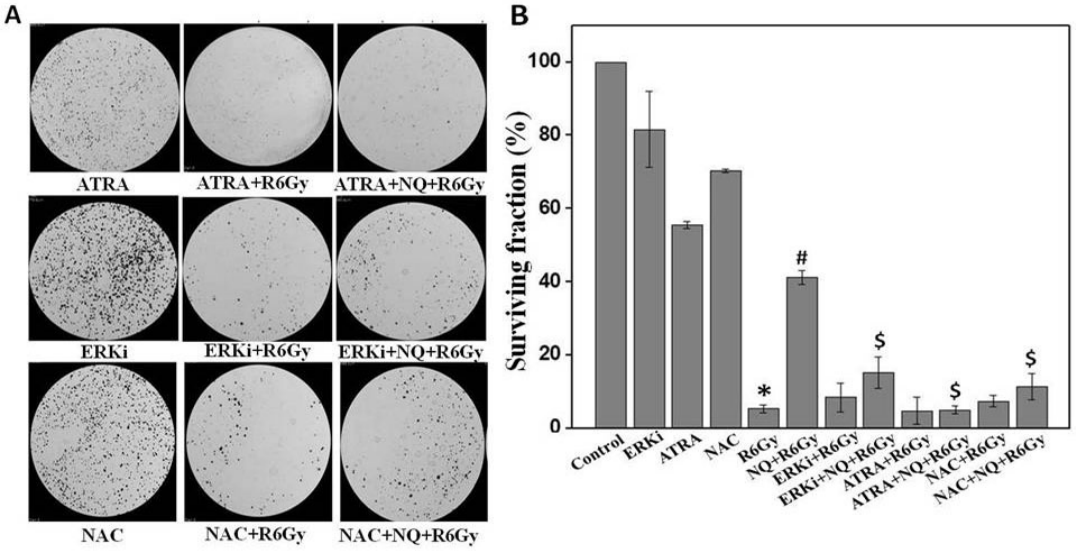

Figure 4. Thiol antioxidant and ERK/Nrf2 pathway inhibitors abrogated the NQ mediated radioprotection. INT 407 cells were pretreated with NQ or NAC (1 mM) or ERKi (PD98059, 5 μM) or ATRA (Nrf2i, 1 μM) for 2 h before being exposed to IR dose of 6 Gy and cultured for 14 days to monitor the clonogenic potential. Vehicle treated cells served as control. Visible colonies were enumerated after staining with crystal violet. **(A)** Pictures shown represent the observed stained colonies from the respective groups. **(B)** Graphed is the percent surviving fraction obtained after enumerating the visible colonies. Each bar represents mean ± S.E.M. from three replicates and two such independent experiments were carried out. The statistical analysis was done using analysis of variance followed by post hoc analysis using Schiffe's test. *$p < 0.01$, as compared to control, # refers to $p < 0.01$ as compared to irradiated group and $ refers to $p < 0.01$ as compared to NQ + IR group.

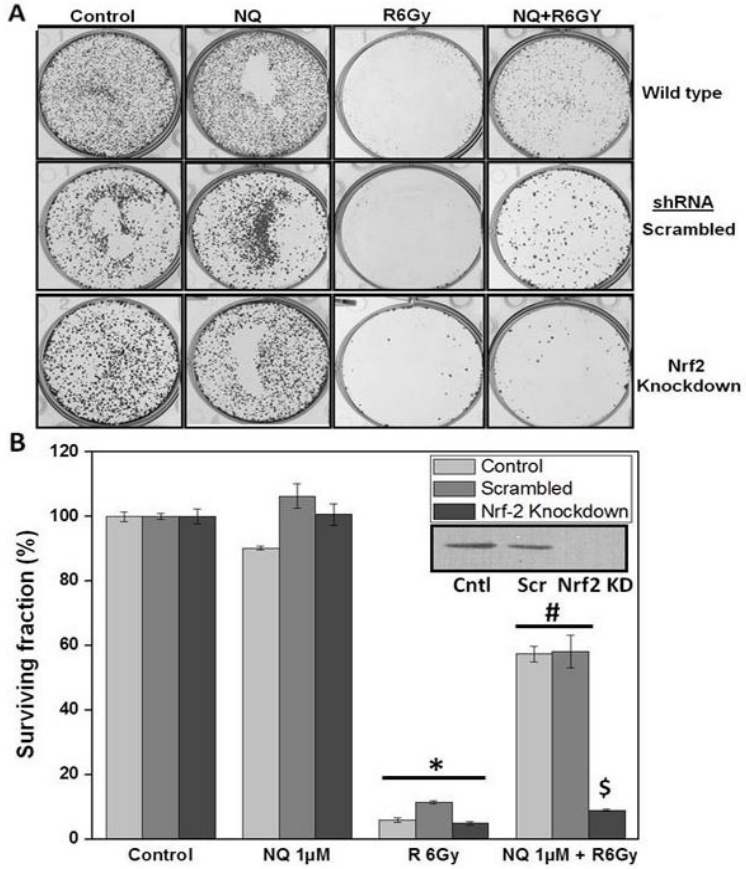

Figure 5. Knocking down Nrf2 reversed the radioprotection offered by NQ in INT 407 cells. INT 407 cells were transfected with scrambled shRNA or Nrf2 shRNA plasmid and were incubated for 24 h. Transfected cells were treated with vehicle or NQ 1 μM for 2 h followed by exposure to IR dose of 6 Gy. Cells were further cultured for 14 days to monitor the clonogenic potential. Visible colonies were enumerated after staining with crystal violet. **(A)** Pictures shown represent the observed stained colonies from the respective groups. **(B)** Graphed is the percent surviving fraction obtained after enumerating the visible colonies. Each bar represents mean ± S.E.M. from three replicates and two such independent experiments were carried out. The statistical analysis was done using analysis of variance followed by post hoc analysis using Schiffe's test. *$p < 0.01$, as compared to control, # refers to $p < 0.01$ as compared to irradiated group and $ refers to $p < 0.01$ as compared to NQ + IR group.

responses. Under normal physiological conditions, cells are exposed to multiple exogenous and endogenous oxidative stresses (*25*). Cells are equipped with network of antioxidant enzymes that plays a pivotal role in detoxification of ROS. The equilibrium between generation and elimination of ROS maintains the cellular redox homeostasis which acts to resist against various stress conditions. ROS are known to play dual role depending upon the magnitude of generation (*26*). High levels of ROS induce activation of apoptotic pathway whereas low levels of ROS initiate the induction of cytoprotective responses. The functional status of cellular antioxidant systems and the redox-sensitive survival signaling pathways can significantly influence the cell-fate against deleterious stimuli (*27*). Therefore, perturbation of cellular redox status by inducing mild oxidative stress may lead to activation of redox sensitive pro-survival pathways. Mild oxidative stress may thus serve as an amenable strategy to develop novel redox based therapeutics/preventive agents.

As radiation induced toxicity is mediated by generation of ROS, employing a potent antioxidant as agents to act as radioprotector seem to be the promising strategy. Though most of the agents investigated have been associated with off target effects and none have been approved by FDA to be used as radioprotector except amifostine (*28-30*). Amifostine has been approved to be used along with cisplatin in radiotherapy however it's not been approved for use outside clinic as it is also associated with the induction of multiple side effects, including nausea, vomiting, sneezing and hypertension (*31-34*). Thus multiple efforts have been undertaken to identify novel targets that could be exploited to develop novel radioprotective agents. Although plethora of agents has been shown to protect against IR induced hematopoietic syndrome, few have been documented to protect against IR induced GI syndrome (*2,35*). Radiation induced GI syndrome is one of the major cause of health impairments or in extreme cases death characterised by denudated villi and apoptosis in crypts. Humans are at higher risk of developing acute and chronic symptoms known as intestine mucositis including pain, ulceration, vomiting and diarrhoea at doses above 2 Gy (*36,37*). ISC present at the base of the crypts called as columnar base cells are responsible for maintaining the homeostasis in intestine. Exposure to ionising radiation induces apoptosis in the ISC which abolishes the regeneration of the denudated villi and marks the rapid onset of GI syndrome (*8,38-40*). Multiple strategies have been employed to investigate the novel targets as well as agents with potential to ameliorate or mitigate the radiation induced damages with lesser side effects.

NQ, a pro-oxidant, has been shown to protect against radiation induced hematopoietic syndrome and its potential to act as anti-inflammatory agent by activating Nrf2 pathway in lymphocytes. Present study was undertaken to investigate the potential of NQ to protect at high doses of IR using *in vitro* model, human intestinal epithelial cell, INT 407. Interestingly, NQ protected against radiation induced cell death in INT 407 cells (Figure 1). Since NQ is a known pro-oxidant, it perturbs the cellular redox status of the INT 407 cells by increasing the basal level of ROS and depleting GSH/GSSG (Figure 2). Higher redox potential and electrophilic nature of quinones imparts high affinity for cellular nucleophiles like thiols of cysteine group present in proteins and glutathione contributing to its biological activity (*41*). Perturbation in cellular redox status is known to activate redox sensitive pro-survival transcription factor Nrf2 and its dependent cytoprotective genes (*42-45*). Activation of Nrf2 has been shown to maintain intestine homeostasis, protect against radiation induced DNA strand breaks in intestine and to regulate the intestinal stem cell proliferation. Activation of Nrf2 results in elevated levels of cytoprotective genes which provide an adaptive response to protect against radiation induced damage (*17,46,47*). NQ induced the activation of ERK/Nrf2 axis in time and concentration dependent manner in INT 407 cells (Figure 3). Captivatingly, thiol antioxidant and inhibitors of ERK/Nrf2 pathway abrogated NQ mediated radioprotection contemplating the imperative role of Nrf2 (Figure 4). Further, knock down studies using shRNA of Nrf2 corroborated the significance of activation of Nrf2 pathway as underlying mechanism of NQ induced protection against IR induced GI syndrome (Figure 5). Further investigations in *in vivo* model system are required to corroborate the present findings.

To best of our knowledge, this is the first report illustrating the salutary effect of a pro-oxidant in the perspective of radiation induced GI syndrome where it played a protective role by inhibiting radiation induced cell death. Since, NQ has a profound anti-cancer activity; it may find application in increasing the therapeutic ratio of abdominal irradiation in GI malignancies. These findings further provide evidence for the putative role of Nrf2 as a key player in developing novel radioprotective agents.

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