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TICKLING OF CELLULAR REDOX STATUS: A NOVEL STRATEGY TO DEVELOP POTENT RADIOPROTECTIVE AND ANTI-INFLAMMATORY AGENTS

Dr. Lokesh Gambhir*

Department of Life Sciences, Shri Guru Ram Rai University, Dehradun, Uttarakhand, India.

***Corresponding Author: Dr. Lokesh Gambhir**
Department of Life Sciences, Shri Guru Ram Rai University, Dehradun, Uttarakhand, India.

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ABSTRACT
Multiple strategies have been investigated to develop novel radioprotectors. But none of them have been approved by FDA as putative agent outside clinic. Thus, a dire need exists to postulate a new strategy that can yield a spectrum of novel compounds with potential to ameliorate radiation insult. Since, inflammation mediates the pathogenesis of radiation injury, a putative agent which is able to elicit anti-inflammatory and cytoprotective response in the cell shall be a putative agent. Cellular redox homeostasis is essential for maintaining viability and normal physiological functions of cell. To maintain the redox balance cells are equipped with a network of antioxidant enzymes that can detoxify the induced oxidative stress. The present review highlights the perturbation of cellular redox status by employing redox modifiers like prooxidants as an amenable strategy for developing novel radioprotectors and anti-inflammatory agents. Prooxidants lead to the activation of redox sensitive immunoregulatory and cytoprotective transcription factor Nrf2. Activation of Nrf2 leads to production of cytoprotective antioxidants to combat radiation induced oxidative damage, anti-inflammatory proteins and downregulation of NF-κB to combat inflammation.

KEYWORDS: Nrf2, oxidative stress, radiation protection, NF-κB.

INTRODUCTION
Advancement in technology has driven an increase in peaceful use of nuclear energy and radio-isotopes in the fields of nuclear industry (power production), medical imaging, diagnostics, food preservation, sanitization, sludge hygienization, smoke detectors, radio-carbon dating, treatment of cancer by external beam therapy or brachytherapy, research and many other industries. Increasing use of ionizing radiation (IR) in different human endeavours is also associated with increased risk of unwanted exposure to toxic doses of IR during planned as well as unplanned settings. Besides these, nuclear disasters or use of dirty bomb by terrorists can also put humans to high risk of exposure to IR.

Although there is an increased use of radiation in our day to day life, it has been considered an enigma to the general public and the use of radiation for therapeutic or other uses has also been associated with some cynicism. The deleterious effects of radiation on human health became evident after study of the health related abnormalities in Japanese atom bomb survivors.[1] Since then understanding the mechanisms of radiation induced injury to cells has been the prime research area of radiation biologists and radiation chemists.[2] It is estimated that half of all cancer patients will receive radiotherapy during the course of their treatment.[3] Under these situations, elevated doses of IR cause damage to normal tissues surrounding the tumor, primarily the hematopoietic system and the gastrointestinal tract, thus inducing detrimental side effects and reducing the therapeutic gain. Unplanned exposure can happen at low doses during occupational exposure or it may reach high doses due to the accidents at nuclear power plants,[4] exposure to contaminated waste,[5] following nuclear "dirty bomb"[5] or due to industrial accidents during mining, milling and processing of radioactive materials . There is a risk of exposure to high doses of radiation not only for radiation workers but also for personnel participating in the emergency response.

Biological effects of ionizing radiation
Deleterious effects of IR on biomolecules are mediated by two distinct phenomenon, indirect effect and direct effect (Fig.1.). Direct effect of IR is mediated via deposition of energy directly on macromolecules like DNA or other cellular components like lipids or proteins which are critical for the survival of the cell. Direct deposition of energy leads to ionization of both the nitrogenous bases and sugar to generate single strand breaks and tandem DNA damage.[6] Indirect effect accounts for 70% of the damage to cells and they are mediated via IR induced generation of reactive oxygen

species (ROS) due to radiolysis of water molecules in the cells. Radiolysis of water molecules results in the formation of hydrogen radicals, hydroxyl radicals, hydroperoxyl radicals and superoxide radicals.[7]

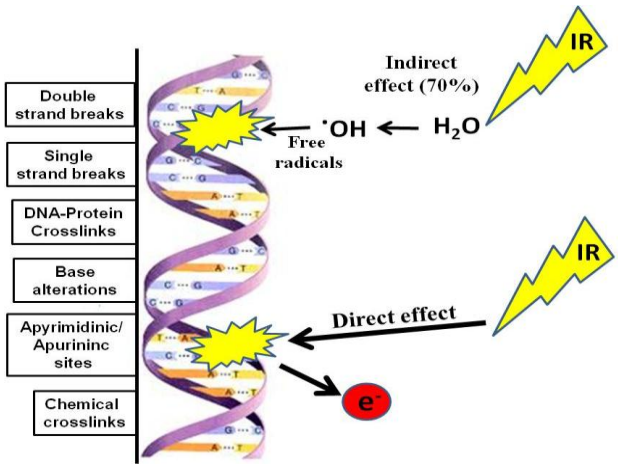

Fig. 1: Radiation induced DNA damage via direct and indirect effects.

The free radicals formed as a result of radiolysis of water have very short life and are capable of interacting with biomolecules like DNA, proteins and lipids in their vicinity. These interactions initiate a cascade of signaling molecules involved in sensing and repair of DNA damage, protein-kinases, cell cycle check points and cell survival or apoptosis. This can either result in cell cycle arrest so as to facilitate repair of radiation induced damage or induce apoptosis.[8] The pathological processes of radiation injury is initiated immediately after radiation exposure, but the clinical and histological features may not become apparent for weeks, months or even years after exposure. If the dose of radiation involved is large enough, acute doses may result in effects which can manifest themselves within a period of hours or days. These conditions are referred as acute radiation syndrome. The acute radiation syndrome occurs after whole-body irradiation (WBI) at doses in excess of 1Gy delivered at a relatively high-dose rate. Rapidly proliferating cells are most sensitive to the acute effects of radiation, particularly lympho-hematopoietic cells and intestinal crypt cells. The inherent sensitivity of these cells results in a constellation of clinical syndromes that predominates within a predictable range of doses of WBI exposure.

Radiation Induced Inflammation

Inflammation is a complex localized protective reaction initiated by host cells/tissues of the body to allergic or chemical stimulation, injury and infections. The five classical signs of inflammation are characterized by pain, heat, redness, swelling and loss of function. Initiation of inflammation is a multifactorial signaling process that result in increased movement of leukocytes, protein and fluids into the affected region due to dilated blood vessels.[9] Outcome of inflammation is mediated by secretion of chemical mediators from cells like mast cells, platelets, neutrophils and monocytes/macrophages. These mediators are termed as pro-inflammatory factors that determine the severity of inflammation.[10]

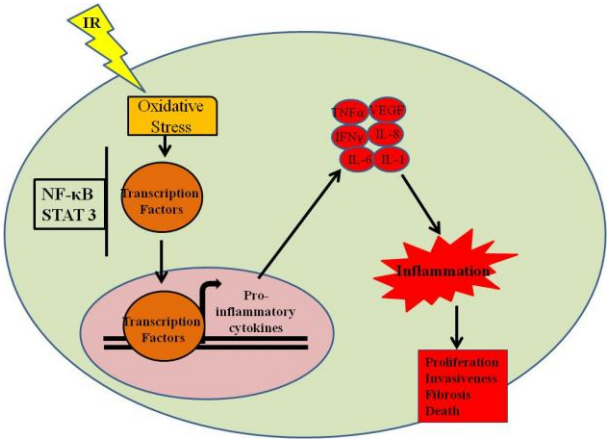

Fig. 2: Radiation induced inflammation.

Radiation exposure induces activation of key immunoregulatory transcription factors. Increase in pro-inflammatory cytokines and adhesion molecules induces inflammation and its associated effects.

Whole body irradiation results in systemic inflammation that also has a profound effect on the repair of radiation injury in different tissues. Apart from direct and indirect damage caused by radiation, inflammation also significantly contributes towards tissue injury (Fig.2.). It is well known that chronic inflammation can amplify the damage inflicted to different tissues by exogenous agents.[11] Radiation-induced inflammatory response is initiated by the production of reactive oxygen/nitrate species, induction of apoptosis, clonogenic cell death, mucosal breakdown and increased transcription of several pro-inflammatory cytokines and chemokines by recruited immune cells and residing cells, depending on the severity of damage.[12] IR induced inflammatory process is initiated by the production of pro-inflammatory mediators such as IL-1β, IL-6, IL-8, CXCL-1, CXCL-2 and TNF-α within minutes to hours. Increased levels of TNF-α and IL-1 have been reported after irradiation of alveolar macrophages or tumour cells.[13] Exposure to X-ray results in over-production of IL-6 and IL-8 in keratinocytes, fibroblasts and glioma cells. IR induced inflammatory responses also play a vital role in pathogenesis of late effects of IR exposure. The IR induced fibrotic tissue remodelling is a multicellular process regulated by different cytokines such as TGF-β1, TNF-α, IL-1, IL-4 and IL-13; chemokines such as MCP-1 and MIP-1β.[14]

Infection associated inflammation is the primary cause of death during acute radiation syndromes. The affected patients with bone marrow aplasia experience reduced defence against exogenous and endogenous pathogens leading to infection and inflammation, and consequently suffer from invasive infection and organ dysfunction resulting in death.[15] IR exposure leads to the activation

of immunoregulatory transcription factors which control the expression of numerous immune mediators involved in cancer promotion and progression. Thus, targeting the IR induced inflammatory signaling pathways may improve the clinical outcomes of radiotherapy.[16,17] It has been observed that suppression of pro-inflammatory transcription factor NF-κB by over-expression of IκBα leads to sensitization of human glioblastoma, fibroblast and intestinal epithelial cells to radiation.[18] Curcumin has been shown to down-regulate NF-κB expression and STAT-3 phosphorylation resulting in better therapeutic gain.[19] Thus, targeting a regulator of which is critical for regulation of inflammation and IR induced damage may serve in developing novel dual role drugs. Over the past decade, cellular redox has been identified impervious to radiation induced responses and inflammation.

Cellular Redox Homeostasis

Cellular redox status is determined by the ability of a cell to maintain the balance between the magnitude of generated oxidative stress and the rate of its detoxification.[20] Maintaining the redox balance is important for proper function and responses of cells. Any disturbance in the redox homeostasis induces oxidative stress mediated signaling cascade that could lead to cell death or induce adaptive survival responses. Outcome of perturbation in the redox balance depends on the magnitude of oxidative stress induced inside the cell.[21]

The intracellular "redox homeostasis" or "redox buffering" capacity is maintained primarily by glutathione (GSH oxidized / reduced) and thioredoxin (TRX oxidized / reduced) redox couples. GSH/GSSG ratio represents the major cellular redox buffer and it is therefore used as an indicator of the redox environment of the cell.[22,23] Basal levels of reactive oxygen species (ROS) are endogenously produced in the mitochondria due to partial reduction of oxygen, inflammatory reactions and enzyme linked reactions (NAPH oxidase, Xanthine oxidase, cytochrome c oxidase).[24]

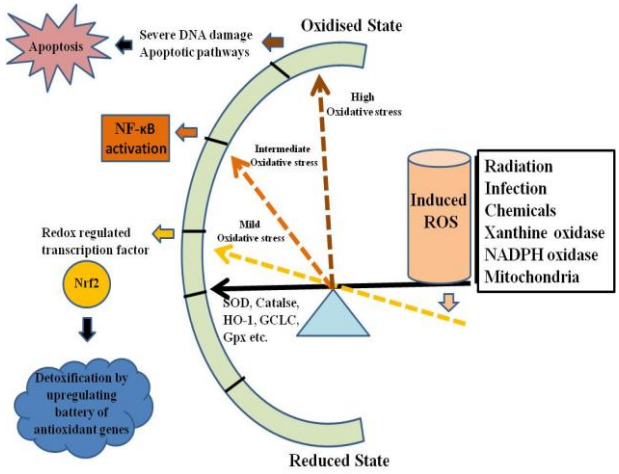

Fig. 3: Perturbation of cellular redox homeostasis.

Induction of mild oxidative stress activates redox sensitive pro-survival pathways like Nrf2 that protects against the oxidative damage. High oxidative stress leads to induction of apoptosis.

Being highly reactive in nature, ROS, when produced at high rate can interact in a nonspecific manner with a wide range of macromolecules including proteins, carbohydrates, lipids and DNA leading to the alteration and impairment of function of cellular components leading to apoptosis (Fig. 3.).[25] However, generation of low levels of ROS can activate several redox-sensitive pro-survival signaling pathways and function as secondary messengers in the signal transduction.[20] Controlled and organised production of ROS underlines their potential to act as secondary messengers involved in signal transduction from cell surface to nucleus.[26] Scavenging of endogenous ROS can impair normal cellular response like production of cytokines and growth factors by T cells. Redox state of many proteins plays an important role during immune responses.[27] Critical cysteine residues present on proteins act as redox sensors and are prone to oxidation into sulfenic acids or disulphide formation or glutathionylation resulting in the modulation host immune responses.[28] The effect of oxidative stress on functions of these proteins depends on the concentration, duration and location of ROS generated inside the cell. Mild oxidative stress (low levels of ROS) can activate redox-sensitive transcription factors like Nrf2.

Nuclear factor erythroid-2 (NF-E2)-related factor 2–Kelch-like ECH associated protein 1 complex and phase II detoxifying/antioxidant enzymes

The transcription factor, nuclear factor [erythroid-derived 2]-like 2 (Nrf2) also known as heme binding protein 1 was first identified as NF-E2-like basic leucine zipper protein that activates transcription by binding to the tandem NF-E2/AP1 repeat of the β-globin locus control regions. Nrf2 is characterised as Cap`n`Collar (CNC) protein coded by *Nfe2l2* gene on chromosome 2 in humans. It is involved in the control of development of labial and mandibular segment of *Drosophila* by basic leucine zipper DNA binding domain (bZip) homeotic gene.[29] Deletion of Nrf2 renders no effect on phenotypic characters and mortality in mice but imparts sensitivity to oxidative stress.[30,31]

Under normal redox conditions, Nrf2 is present in the cytoplasm at low levels to induce the basal level expression of its downstream cytoprotective genes. Perturbation of cellular redox status leads to activation of Nrf2. Upon activation, Nrf2 dissociates from KEAP-1 and translocates to the nucleus and forms a heterodimer with its co-transcription factor Maf. Nrf2-Maf complex binds to the antioxidant response element (ARE) sequence to regulate the expression of downstream cytoprotective and antioxidant enzymes.[32] The Nrf2 downstream genes include phase II detoxifying enzymes like glutathione S-transferase (GST), NAD(P)H quinone

oxidoreductase-1 (NQO1),[33] and UDP-glucuronosyl transferase (UGT), intracellular cytoprotective proteins like glutamate cysteine ligase (GCL),[34] glutathione peroxidase (GPx),[35] thioredoxin (Trx),[36] thioredoxin reductase (TrxR),[37] peroxiredoxin (Prx),[38] hemeoxygenase-1 (HO-1) and transporters like multidrug resistance-associated protein (MRP).[39] Under homeostatic condition the redox status of a cell is a function of the Nrf2 induced basal level expression of antioxidant enzymes and cytoprotective proteins. However, regulation of Nrf2 under stress condition determines the fate of a cell.

Generation of mild oxidative stress is known to activate Nrf2 by abrogating KEAP-1 induced degradation of Nrf2. KEAP-1 is a redox sensitive protein due to presence of multiple sulfhydryl groups of more than 20 critical intrinsic cysteine residues.[40] Mutation at cys151 in KEAP-1 abrogated the activation of Nrf2 by sulforaphane and *tert*-butylhydroquinone. Interestingly, it had no effect on KEAP-1:Nrf2 binding implicating that cys151 though critical for Nrf2 activation by putative inducers but is not obligatory for Nrf2 binding. Intriguingly, cys151 KEAP-1 mutant cells showed normal activation of Nrf2 by arsenite.[41] Along with cys151, cys273 and cys288 were identified as critical cysteine residues by employing *in vitro* alkylation and *in vivo* site-directed mutagenesis. Further, mutations at cys273 and cys288 subdued KEAP-1 mediated repression of Nrf2 highlighting that cys273 and cys288 are essential for Nrf2 activation.[42] Modification of cysteine residues impede the E3 ligase activity resulting in detachment of Cul3-E3 complex from KEAP-1 thereby abrogating Nrf2 degradation. However, KEAP-1 remains bound to Nrf2 at ETGE motif thereby causing translocation of free Nrf2 to the nucleus and expression of its dependent genes.[43] Alternatively, abated Cul3 interaction resulting from polyubiquitination of KEAP-1 at lys63 has also been attributed to dissociation of Nrf2 from KEAP-1.[44] Apart from redox dependent mechanism, multiple studies have also highlighted the redox independent activation of Nrf2. Post translational modification also governs the Nrf2 activation. Nrf2 contains multiple serine, threonine and tyrosine residues which can serve as potential sites for phosphorylation. Different kinases have been identified including protein kinase C (PKC), mitogen-activated protein kinases (MAPK), phosphatidylinositol 3-kinase (PI3K) and RNA-dependant protein kinase-like endoplasmic reticulum kinase (PERK).[45]

Apart from the cytoprotective nature of Nrf2, its role as a redox sensitive anti-inflammatory transcription factor has been well documented. Recent reports reveal an important role of Nrf2 in modulating immune responses and ameliorating immune disorders. Nrf2 knockout mice showed enhanced bronchial inflammation,[46] prolonged inflammation during cutaneous wound healing,[47] high susceptibility for lupus like autoimmune syndrome, enhanced lymphocyte proliferation and impaired redox status.[48] Stimulated lymphocytes from Nrf2 deficient mice showed higher secretion of Th2 cytokines like IL-4 and IL-13.[46] Further, Nrf2 deficient mice showed higher mortality to septic shock caused by lipopolysaccharide or cecal ligation or puncture.[49] Nrf2 dependent protein, HO-1 and its degradation product carbon monoxide, have been reported to show anti-inflammatory effects by inhibiting pro-inflammatory cytokine production, leukocyte migration, adhesion and suppressed LPS induced production of tumour necrosis factor-*a* (TNF-*a*) and nitric oxide (NO) in murine macrophages.[50] Several studies have shown an indispensable role of Nrf2 and its dependent gene HO-1 in controlling inflammatory responses via regulation of cytokines and pro-inflammatory proteins and its deficiency was shown to increase susceptibility to inflammatory disorders.[51] Several investigators have highlighted the role of KEAP-1/Nrf2 pathway in regulating NF-κB activation as well as NF-κB mediated inflammatory response.[52]

Cross Talk between Nrf2 and NF-κB

Apart from the anti-inflammatory action of Nrf2 dependent genes, activation of Nrf2 has been shown to suppress activation of NF-κB there by inhibiting inflammatory reactions. Multiple studies have highlighted the cross talk between Nrf2 and NF-κB as prime target for development of novel anti-inflammatory agents. Several anti-inflammatory or anti-carcinogenic phytochemicals suppress NF-κB signaling and activate the Nrf2-ARE pathway. Nrf2-deficient mice, subjected to a moderately severe head injury, show a greater cerebral NF-κB activation compared with their wild-type Nrf2 counterparts[53] while Nrf2 over expression suppresses NF-κB- DNA binding activity.[54] Cinnamaldehyde activates Nrf2 and inhibits the degradation of IκBα leading to suppression of NF-κB pathway.[55] Curcumin, a polyphenol, was shown to activate Nrf2 which further mediated the modulation of expression and transactivation of NF-κB.[56] Kim et al showed the ability of KEAP-1 to suppress NF-κB pathway by inhibiting phosphorylation and inducing degradation of IKKβ.[57] These studies clearly demonstrate the potential of the cross talk between these two transcription factors for therapeutic interventions.

Cellular Redox modulation as novel strategy for development of radioprotective and anti-inflammatory compounds

Ionizing radiation mediated tissue injury is also mediated through systemic inflammation.[58,59] Work on development of radioprotective agents started more than six decades ago and identification of radioprotective agents has been an important goal for radiation oncologists as well as radiation biologists. The potential application of prophylactic agents is to protect normal tissues, but not tumors, during planned exposure settings like cancer radiotherapy. However, therapeutic agents find applications mainly during accidental exposures. Multiple strategies have been employed to develop putative agents that can protect against radiation induced

damage like free radical scavengers, inhibitors of radiation induced cell death signaling pathways, cell cycle modulators, cytokines, growth factors and activators of pro-survival pathways (Fig.4.).

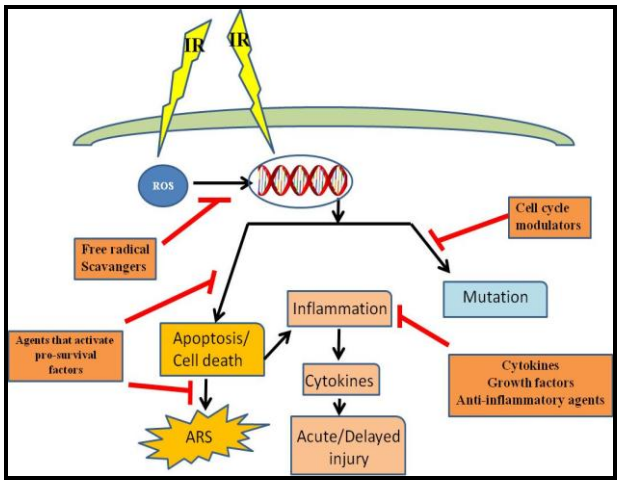

Fig. 4: Strategies employed to develop a potent radioprotector.

Since radiation induced damages are mediated by the induction of free radicals, employing a free radical scavenger was the first amenable strategy. Several studies using free radical scavengers such as N-acetyl cysteine, glutathione, β-mercaptoethylamine (cysteamine, MEA), propylgallate, vitamin-E & vitamin-C showed their ability to protect mice against IR induced toxicity.[60,61] Endogenous antioxidants such as superoxide dismutase, catalase, peroxiredoxins, thioredoxin and glutathione have been used to develop potent radioprotectors.[62] Thiol group containing molecules have been investigated extensively due to their ability to donate reducing equivalents to unstable molecules such as reactive oxygen species.[63] Apart from antioxidants, other agents have been investigated including granulocyte colony-stimulating factor (G-CSF) which improved bone marrow recovery and reduced WBI induced lethality.[64] Keratinocyte growth factor (KGF) is another radio-mitigator and it has been shown to stimulate a number of processes such as differentiation, DNA repair, as well as scavenging reactive oxygen species.[65] KGF has been recently approved by FDA for mitigating oral mucositis.[66] Activation of NF-kB signaling has been exploited to induce radioprotective cytokines and thereby suppress apoptosis.[67] CBLB502, a polypeptide derived from *Salmonella* flagellin, which binds to toll-like receptor 5 and subsequently activates NF-kB signaling was shown to protect against WBI induced hematopoietic and GI syndrome associated mortality while it did not alter radiosensitivity of the tumors.[68] Despite the potent efficacy of several investigated agents, none of them could reach clinic to be used as general radioprotector leaving this area of research wide open to develop newer agents which can surpass some of the drawbacks of previously developed radioprotective agents. The strategy of using antioxidants/free radical scavengers as radioprotectors has not yielded expected results in the clinic. This could be due to need for its presence systemically in cells with uniform distribution among the cellular compartments in sufficient concentrations to nullify the toxic effects of ROS. Achieving such high concentration of antioxidant throughout the living system to effectively detoxify free radicals is less plausible. Further, the efficacy of antioxidants to neutralize free radicals also depends on its rate of reaction with the free radicals. Thus, antioxidant property alone may not be sufficient to neutralize radiation induced toxicity.

To pave a new path for developing novel agents, it is hypothesized that activation of pro-survival pathways by employing a pro-oxidant prior to radiation exposure may prime the cells to counterbalance the radiation insult. Activation of antioxidant, anti-inflammatory and cytoprotective machinery under the regulation of Nrf2 would prepare the cell to combat against radiation induced oxidative stress. This is in contrary to the classical belief that radical scavengers could be potent radioprotectors. Multiple studies have highlighted the potential of pro-oxidants to protect against oxidative stress induced cellular damage.[69] Plumbagin, a naphthoquinone derivative and a pro-oxidant, was shown to protect oxidative stress induced spinal injury.[70] Further, sulforaphane, a redox modifier and potent Nrf2 activator agent, has been shown to protect against oxidative stress induced focal cerebral ischemia, brain inflammation, ischemia and acute renal failure.[71] Similarly, 15d-PGJ(2) protected astrocytes against Mn-induced inflammation and oxidative stress by modulating the activation of the NF-κB and Nrf2 pathways.[72] 1, 4-naphthoquinone (NQ), a well-known pro-oxidant, offered protection against radiation induced hematopoietic syndrome in mouse model via activation of Nrf2 pathway. Further, 1,4- naphthoquinone (NQ) was shown to protect INT 407 cells from radiation insult. Since NQ was able to activate Nrf2,[69] an immunoregulatory transcription factor and a negative regulator of NF-κB, Gambhir et al showed the anti-inflammatory effects of 1,4 NQ in innate and adaptive immune responses both in vitro and in vivo. NQ induced KEAP-1 mediated degradation of IKKβ leading to abrogation of NF-κB pathway. These observations further contemplate the hypothesis of perturbing cellular redox as a novel strategy for developing anti-inflammatory drugs.

CONCLUSION

Under normal physiological conditions, cells are exposed to multiple exogenous and endogenous oxidative stressors and maintenance of cellular redox homeostasis is very important to maintain cell viability and normal physiological responses.[22] To maintain redox homeostasis and to counter oxidative stress, cells are equipped with network of antioxidant enzymes that plays a pivotal role in detoxification of ROS.[73] ROS are known to play dual role depending upon the magnitude of generation. It is well accepted that high levels of ROS

induce activation of apoptotic pathway whereas low levels of ROS initiate the induction of cytoprotective responses.[74] Therefore, perturbation of cellular redox status by inducing mild oxidative stress can lead to activation of redox sensitive pro-survival pathways which trigger the cytoprotective and anti-inflammatory responses. Induction of mild oxidative stress may thus serve one of the amenable strategies to develop novel redox based therapeutics/preventive agents (Fig.5.).

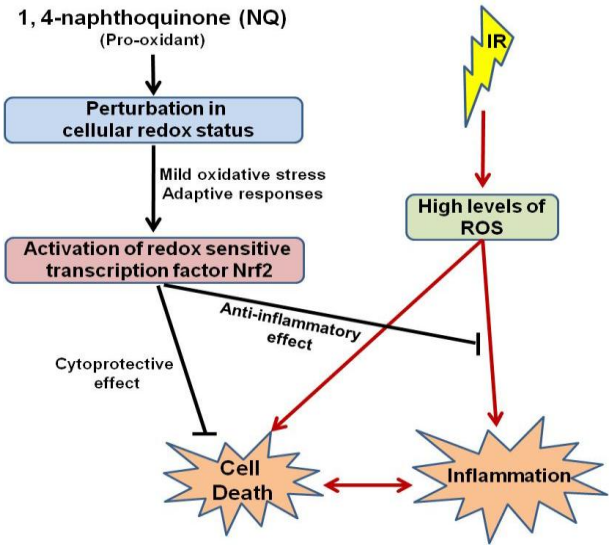

Fig. 5: Hypothesis of pro-oxidants activating Nrf2 thereby leading to radioprotection.

Multiple strategies have been employed by different investigators to identify the novel targets and agents with potential to ameliorate IR induced syndromes.[68,75] Though most of these agents investigated thus far have not yielded expected results and none of them are approved by FDA to be used as radioprotector. Amifostine has been approved by FDA for a specific indication to be used in Head & Neck cancer patients undergoing radiotherapy. However, it is not approved as a general radioprotector as it is also associated with the induction of multiple side effects including nausea, vomiting, sneezing and hypertension. In the present review, a novel strategy is projected of using cellular redox modifiers to induce Nrf2 cytoprotective pathways for mitigation of radiation induced injury and ameliorating inflammatory responses.

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