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H_2O_2 generation by BCG induces the cellular oxidative stress response required for BCG's direct effects on urothelial carcinoma tumor biology

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Abstract

INTRODUCTION—Exposure of urothelial carcinoma (UC) cells to Bacille Calmette Guerin (BCG) affects cellular redox status and tumor cell biology but mechanism(s) remains unclear. This study examined free radical production by BCG, and in tumor cells in response to BCG, using global profiling of Reactive oxygen species/reactive nitrogen species (ROS/RNS). The relationship between free radical generation and downstream cellular events was evaluated.

MATERIALS AND METHODS—Using fluorescent probes, global profiling of ROS/RNS was carried out in Heat killed (hk) BCG, viable BCG, and in two UC cell lines post BCG exposure (253J and T24). Inhibition of BCG internalization and pharmacologic scavenging of H_2O_2 was studied for their effect on cellular ROS/RNS generation and various physiological end points.

RESULTS—Viable BCG produced H_2O_2 (Hydrogen peroxide) and O_2^- (Superoxides) but did not show NO (Nitric oxide) generation. Loss of viability decreased production of H_2O_2 by 50% compared to viable BCG. BCG internalization was necessary for BCG induced ROS/RNS generation in UC cells. Pharmacologic H_2O_2 scavenging reversed the ROS/RNS mediated signaling in UC cells. BCG dependent alterations in tumor biology including intracellular signaling, gene expression and cytotoxicity were dependent on free radical generation.

CONCLUSIONS—This study demonstrates the importance of free radical generation by BCG, and intracellular generation of Cellular oxidative stress (COS), on the UC cell response to BCG. Manipulation of the BCG induced COS represents a potential target for increasing BCG efficacy.

Keywords

Bladder cancer; BCG; H_2O_2; Oxidative stress; global profiling

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Introduction

The intravesical administration of the attenuated *Mycobacterium* Bacille Calmette Guerin is standard of care treatment for high risk, non-muscle invasive bladder cancer. [1–2] Post BCG exposure, UC cells undergoes complex changes in redox status, intracellular signaling and gene expression. BCG internalization is associated with the downstream activation of multiple stress responsive pathways involving NF-κB, NrF2 and CEBP. These signaling pathways direct the transactivation of genes which subsequently contribute to alterations in tumor biology and phenotype. In addition to this direct effect on UC cells, internalization of BCG by UC cells triggers the expression of white blood cell receptors and the release of cytokines and chemokines. These effects combine to recruit and target various types of immune cells into the bladder. Activated phagocytes and cytokines leads to the differentiation of $CD4^+$ T cells into TH1 and/or TH2 type immune response.[3] Through a combination of both direct and systemic effects, the interaction between UC cell and BCG plays a critical role in the ultimate response to treatment.

Previous studies have shown that BCG, both live and lyophilized affects cellular ROS generation and oxidative stress in cancer cells.[4–6] However, the precise mechanism through which BCG generates oxidative stress was not reported and the relationship between BCG induced oxidative stress and BCG dependent cellular events has not been investigated. This study evaluated ROS/RNS generation by BCG and BCG exposed tumor cells. The role of BCG internalization, BCG viability and intracellular H_2O_2 levels in mediating cellular changes in ROS/RNS and downstream cellular events were evaluated. Our results show that viable BCG acts as H_2O_2 generator. BCG internalization and generation of intracellular H_2O_2 is required for changes in cellular redox status, ROS/RNS production and the direct biologic response of UC cells to BCG. The identification of H_2O_2 and O_2^- production by BCG is a novel and previously unreported finding. BCG production of H_2O_2 and O_2^- contributes, if not playing a central role, to the known BCG-induced alterations in cellular redox status and the associated changes in UC cell biology.

Materials and Methods

2.1 Cell Lines

The human UC cell line T24 was obtained from American Type culture collection Culture (Rockville, MD). The 253J cell line was a kind gift of Dr. Richard Williams (University of Iowa). Cells were maintained as described earlier.[7]

2.2 Bacillus Calmette-Guerin (BCG)

TICE BCG and HkBCG were prepared and used as described earlier.[8]

2.3 Real time measurement of ROS and RNS in bacterial cells

Fluorescence probes were used for detection of ROS/RNS as described earlier.[9] Coumarin 7-boronic acid (CBA) was synthesized as described earlier.[9] Amplex® Red (AR) (10-acetyl-3,7-dihydroxyphenoxazine)/horseradish peroxidase were purchased from Cayman Chemical. CBA and AR were used for H_2O_2 detection. Hydroethidine (HE) was purchased

from Invitrogen for O_2^- detection. Diaminofluorescein–2 diacetate (DAF-2DA) was purchased from EMD calbiochem for NO detection. For detection of ROS/RNS, equal number of reconstituted BCG and hkBCG were suspended in sterile DPBS. For measurements using the fluorescence plate reader, immediately after the addition of DPBS containing the probe CBA (25 μM), AR (50 μM), HE (40 μM) and DAF-2DA (10 μM), the plate with cells was placed in the plate reader pre warmed to 37°C and was read for 1h.

2.4 Fluorescent plate reader

Total fluorescence intensities were obtained using Beckman Coulter DTX-880 plate reader. The instrument was kept at 37°C during the measurements and fluorescence intensity read from bottom of each well was integrated over 0.5 s every 90 s. Fluorescence signal was detected at an excitation/emission wavelength for different probes as followed: 320/430 nm for CBA, 535/595 nm for AR, 485/574 nm fro HE, 485/535 nm for DAF-2DA.

2.5 Real Time global profiling of ROS/RNS in UC cells

For long term global profiling, 253J and T24 cells were plated at 1×10^5 cells/well in 24-well plates. 24 h later, BCG (50:1 BCG: cells) was added. Cells were incubated for 1, 3 and 6 days and washed twice with sterile PBS. Intracellular ROS/RNS levels were measured using fluorescence probes as described earlier. To measure ROS/RNS at earlier time points, cells were plated at 1×10^4 cells/well in 96-well plates. 24h later, BCG (50:1 BCG: cells) was added to the cultures. Cells were incubated up to 24h and washed twice with sterile PBS. Intracellular ROS/RNS levels were measured using probes as described earlier. A corresponding control (no BCG) was included at each time point and results were presented as difference (Δ) between corresponding “time correct” control and BCG treated group.

2.6 Effect BCG internalization on global profiling of ROS/RNS in UC cells

Inhibition of the cellular cytoskeleton using the spindle toxin cytochalasin b prevents BCG internalization.[10] To investigate the role of BCG internalization on ROS/RNS generation; cells were plated at 1×10^4 cells/well in 96-well plates. 24h later, cells were pretreated with cytochalasin b (25 μg/ml) for 1h before BCG addition. The cells were incubated for 6h and washed twice with sterile PBS. Intracellular ROS/RNS levels were measured using fluorescence probes as described earlier.

2.7 Effect of ROS scavenger on global profiling of ROS/RNS in UC cells

Ebselen (2-phenyl-1, 2-benzisoselenazol-3(2H)-one) is a potent, cell permeable scavenger of H_2O_2.[11] It functions as a mimic of glutathione peroxidase. To check effect of ebselen on ROS/RNS profiling, cells were plated at 1×10^4 cells/well in 96-well plate. 24h later, cells were pre-treated with ebselen (25 μM) for 1h followed by BCG addition. The cells were incubated for 6h and washed twice with sterile PBS. Intracellular ROS/RNS levels were measured using fluorescence probes as described earlier.

2.8 Effect of ROS scavenger on gene expression

Prior work has demonstrated that UC cell exposure to BCG increases the expression of cell cycle regulatory and immune response genes.[12] The effect of ROS scavenger ebselen on

gene expression in response to BCG was measured after 6 h using qRT-PCR and primers as described earlier.[12]

2.9 Effect of ROS scavenger on Intracellular signaling

The effect of ROS scavenger on signaling pathway activation was measured as described earlier.[8] Cells were untreated or treated with BCG, ebselen and BCG with ebselen for 6h. Luciferase activity was measured using a luciferase assay system (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activities were normalized to protein concentration measured by BCA protein assay kit (Pierce, Rockford, IL).

2.10 LDH release Assay

The effect of ROS scavenger on LDH release in response to BCG after 12h was measured by following manufacturer's instruction (CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit, Promega, Madison, WI).

2.11 Cell Proliferation

BCG is known to exert an antiproliferative effect on UC cells. The effect of ROS scavenger on BCG's anti-proliferative effect was measured using the MTT assay as previously described.[12.] Cytotoxic effects of BCG were also confirmed by using Apo-tox-glo kit (Promega) and results were comparable with MTT.

2.12 Statistical Analysis

All experiments were performed in triplicate at three different time points. qRT PCR data were normalized using housekeeping gene β-actin expression. Data was analyzed using non parametric analysis and ANOVA using graphpad prism 6. Results were considered significant at $p < 0.05$. Graphical representation of the data is shown as the mean ± S.D.

Results

3.1 ROS/RNS generation by BCG

ROS/RNS production by BCG was monitored using fluorogenic probes. BCG showed H_2O_2 and O_2^- production as measured by increase in the fluorescent intensity but did not show NO production (figure 1). Compared to reconstituted BCG, hk BCG showed a 50% decrease in H_2O_2 generation ($p < 0.001$, figure 1). There was no significant difference in O_2^- generation by reconstituted and hk BCG ($p = 0.4$).

3.2 Intracellular real time global profiling of ROS/RNS

UC cells were treated with BCG up to 6 days. Real time global profiling of ROS/RNS generation was measured at 1, 3, 6, 12, and 18h and on days 1, 3 and 6. No significant changes in cellular ROS/RNS were observed beyond 24h (data not shown). When measured using Amplex red fluorogenic dye, both cell lines showed significant early increases in H_2O_2 levels. In T24 cells, the increase in H_2O_2 could be detected by 3h ($p < 0.001$) and remained significantly higher till 12h relative to controls ($p < 0.001$, Figure 2). In 253J cells,

a significant increase in H_2O_2 was observed from 3h ($p < 0.05$) and significantly elevated levels were observed till 18h post BCG exposure ($p < 0.01$, Figure 2).

NO levels were measured using the DAF-2DA probe (Figure 3). BCG exposure resulted in higher levels of NO at 3h with a significant increase observed at 6h in both cell lines ($p < 0.01$) relative to untreated controls. No significant difference in NO levels between control and treated group were observed 6h onwards (T24, $P = 0.57$; 253J, $p = 0.28$).

3.3 Effect of BCG internalization on real time ROS/RNS profiling

Figure 4 shows real time global profiling of ROS/RNS generation in control cells or cells treated with BCG or cytochalasin b and BCG. BCG treated cells demonstrated significantly increased levels of H_2O_2, O_2^- and NO compared to untreated controls. Inhibition of BCG internalization using cytochalasin b resulted in significant decreases in ROS/RNS levels. Relative to BCG alone, cytochalasin b with BCG treatment groups significantly decreased H_2O_2 ($p < 0.05$). O_2^- levels were significantly reduced in the cytochalasin b plus BCG treated group ($p < 0.01$), in both cell lines. NO levels in cytochalasin b plus BCG group showed significantly lower levels compared to BCG alone ($p < 0.05$). ROS/RNS generation profile in the cytochalasin b plus BCG group did not significantly differ from that in the corresponding cytochalasin b alone group ($p = 0.4$).

3.4 Effect of free radical scavenger on real time ROS/RNS global profiling

Pretreatment of UC cells with the cell permeable H_2O_2 scavenger ebselen prior to BCG treatment significantly decreased ROS/RNS generation by UC cells. Figure 5 shows intracellular real time ROS/RNS levels in different treatment groups. H_2O_2, O_2^- and NO levels significantly decreased in the BCG plus ebselen treatment group in both cell lines, relative to BCG alone. ($p < 0.05$ for H_2O_2, $p < 0.001$ for O_2^- and NO). ROS/RNS generation profile in the ebselen plus BCG group did not significantly differ from that in the corresponding ebselen alone group ($p = 0.7$).

3.5 Effect of free radical scavenger on Gene Expression

Ebselen significantly decreased the response of UC cells to BCG as measured gene expression of several immune response genes. Figure 6 demonstrates the effect of ebselen on the UC cell expression of a panel of 8 BCG responsive genes as measured by q-rtPCR. Ebselen in combination with BCG reduced UC cell expression of all measured genes with 7 of 8 and 8 of 8 reaching the level of statistical significance in 253J and T24 cells respectively ($p < 0.05$ increase vs. control, $p < 0.05$ decrease vs. BCG).

3.6: Effect of free radical scavenger on intracellular signaling

Exposure of UC cells to BCG for 6 h resulted in the activation of the NF-κB, CEBP and NrF2 intracellular signaling pathways. BCG significantly increased activation of NF-κB, CEBP and NRF2 luciferase reporter construct in both cell lines ($p < 0.01$). Relative to BCG, pretreatment of cells with the ebselen significantly reduced NF-κB ($p < 0.01$), CEBP ($p < 0.05$) and NrF2 ($p < 0.05$) reporter activation. NF-κB, CEBP and NRF2 activation in response to BCG with ebselen treated cells was not significantly different than cells treated with ebselen alone (Figure 7).

3.7 Effect of free radical scavenger on BCG cytotoxicity

Exposure of UC cells to BCG significantly increased LDH release compared to controls in T24 and 253J cells, respectively ($p < 0.001$; figure 8a). Pretreatment with ebselen significantly decreased LDH release relative to BCG alone in both cell lines after 12 h (T24 $p < 0.001$; 253J $p < 0.01$). Of note, ebselen alone resulted in an increase in LDH relative to control cells. Relative to ebeslen alone, LDH release was significantly increased in the BCG + ebselen group ($p < 0.05$).

As measured by the MTT assay, exposure of UC cells to BCG for 24 h significantly inhibited cell proliferation compared to controls in 253J and T24 cells, respectively (T24 $p < 0.005$; 253J $p < 0.05$). Treatment of cells with ebselen prior to BCG treatment inhibited BCG's antiproliferative/cytotoxic effect in 253J and T24 cells ($p < 0.05$, figure 8b).

Discussion

Multiple publications have reported on the direct effects of BCG on the biology of UC cells. BCG adherence to and internalization by UC cells, activation of cellular stress responsive signaling pathways, changes in gene expression and alterations in cellular viability and phenotype characterize the response of UC cells to BCG.[13–15] iNOS activation and NO production in response to BCG are required for downstream cellular events.[16–18] Loss of BCG viability is associated with a reduction in BCG's direct effects.[19–20] While important observations the mechanism through which BCG initiates these changes and how this is correlated with BCG viability, has here-to-fore been undefined.

In what to our knowledge is a novel finding, the current study demonstrates that BCG acts as an H_2O_2 generator. H_2O_2 is member of Reactive oxygen species (ROS) molecules which also includes super oxides and hydroxyl radicals. While H_2O_2 is a signaling molecule, it can also cause significant cellular damage particularly after reacting with reduced iron or copper to generate hydroxyl radicals which are strong oxidizing agents.[21] H_2O_2 production has been reported in many bacterial species including *E.coli*, *Streptoccous* spp., *Pneumococcus spp.*, lactic acid bacteria etc.[22–25] While H_2O_2 is continuously produced in aerobically growing cells as by product of respiration, the main source of hydrogen peroxide production in bacteria is still unclear. In case of *E.coli*, respiratory enzymes were suggested to be source but recently it has been reported that it may be formed by a non-respiratory enzyme or by a cellular metabolite which reacts with oxygen. [22, 26] In case of *Streptococcus sanguinis*, pyruvate oxidase and acetate kinase enzymes have been shown to affect H_2O_2 production.[24] In case of *Streptococcus pyogens*, lactate oxidase was reported to be involved.[24]

It has been previously reported that BCG exposure results in oxidative stress and lipid peroxidation in UC cells.[6] However, the exact mechanism or the source of the oxidative stress was not studied in these prior publications. Based upon the results of this study H_2O_2 production by viable BCG appears to serve as a trigger for cellular oxidative stress and cell damage. Post BCG exposure, UC cells showed increased levels of H_2O_2 till 12-18h. This increase is dependent upon the internalization of viable BCG and the "effective" (non-scavenged) intracellular release of H_2O_2 by BCG. Increased levels of H_2O_2 could set the stage for secondary generation of oxidants involving iNOS, NO and peroxynitrite. Exposure

of cells to H_2O_2 can up regulate iNOS expression and NO production.[27, 28] Several studies have shown that increased iNOS/NO levels in carcinoma cells and laboratory models following intravesical instillation of BCG.[18,19] Prior reports by our group have demonstrated that BCG activates UC cell iNOS expression and NO generation in vitro.[13] The direct effects of BCG on UC biology were dependent upon iNOS expression and NO generation. In the current study intracellular NO levels were temporally related to cellular H_2O_2 production. Along with bacteria induced oxidative stress, cancer cells may also contribute to oxidative stress in response to bacterial infection [29]. It has been postulated that H_2O_2 production by BCG together with eukaryotic NO production can promote peroxynitrite generation leading to further cellular oxidative stress.[30] Oxidative stress also activates signaling pathways involving NF-κB, NrF2 and CEBP, which results in expression of several immune response genes. Previously we have shown that the inhibition of BCG internalization prevents BCG induced UC cell gene expression. Treatment with ebselen which acts as free radical scavenger for H_2O_2, nullified the increase in expression of immune response genes, signaling pathway activation and cytotoxicity, further indicating that the free radical generation post BCG internalization serves as a source of cellular oxidative stress. The relative decrease in H_2O_2 generation by non-viable BCG may explain the loss of therapeutic efficacy associated with the loss of BCG viability. In combination, these findings provide strong evidence that the intracellular generation of H_2O_2 by internalized, viable BCG, is a requisite and early event in initiating the UC response to BCG *in vitro*. It is possible that in the *in vivo* settings, there are additional contributors to cellular oxidative stress (COS), such as activated immune cells are known to produce ROS. Immune cells migrating to sites of BCG therapy may contribute to *in vivo* COS.

H_2O_2 generation by internalized, viable BCG is a potential unifying element in BCG induced oxidative stress and downstream, BCG dependent alterations in tumor biology.

Conclusions

This study demonstrates the importance of free radical generation by BCG, and intracellular generation of Cellular oxidative stress (COS), on the UC cell response to BCG. Pharmacological manipulation of COS in combination with viable or hkBCG provides an alternative strategy for treatment of NMIBC.

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Abbreviations

ANOVA	analysis of variance
BCG	Bacillus Calmette Guérin
hkBCG	Heat killed BCG
CD54	intercellular adhesion molecule 1

CXCL1	chemokine (C-X-C motif) ligand 1
CXCL3	chemokine (C-X-C motif) ligand 3
CCL20	chemokine (C-C motif) ligand 20
DNA	deoxyribonucleic acid
FBS	fetal bovine serum
IL6	interleukin 6
IL8	interleukin 8
iNOS	inducible nitric oxide synthase
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor kappa B
NO	nitric oxide
NRF2	nuclear factor (erythroid-derived 2)-like 2
CEBP	CCAAT-enhancer-binding proteins
P21	cyclin dependent kinase inhibitor p21 (cip1; waf1)
PBS	phosphate buffered saline
RNA	ribonucleic acid
rtPCR	reverse transcriptase polymerase chain reaction
q rtPCR	quantitative reverse transcriptase polymerase chain reaction
UC	urothelial carcinoma
ROS	reactive oxygen species
RNS	reactive nitrogen species
H_2O_2	hydrogen peroxide
O_2^-	superoxides
CBA	Coumarin 7-boronic acid
HE	Hydorethidine
AR	amplex red; 10-acetyl-3,7-dihydroxyphenoxazine
DAF-2DA	Diaminofluorescein–2 diacetate
COS	cellular oxidative stress

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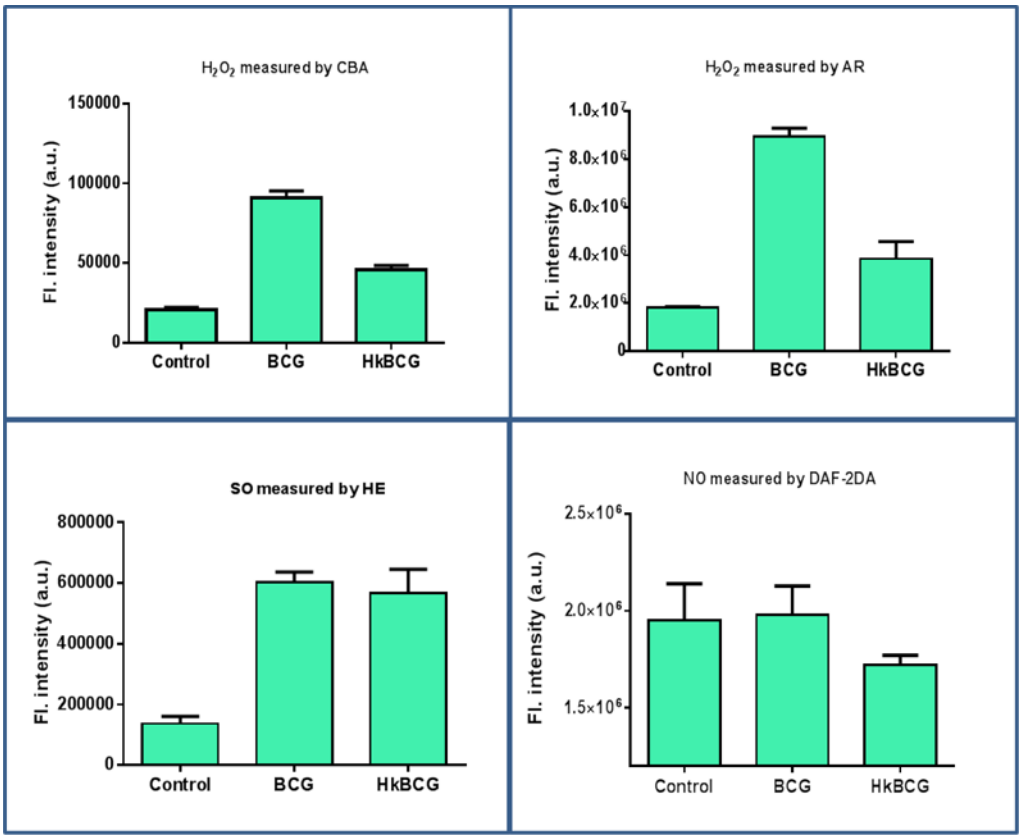


Figure 1.

Global profiling of ROS/RNS generation by BCG. ROS/RNS generation was measured using different fluorescent probes a) H_2O_2 was measured by CBA and AR b) Superoxides were measured by HE c) NO was measured by DAF-2DA. BCG produces H_2O_2 and O_2^- as but do not generate NO. H_2O_2 production by BCG is affected by viability. Hk BCG produced decreased amount of H_2O_2 compared to freshly reconstituted BCG ($p < 0.001$). O_2^- production was not affected by viability as there was no significant difference in viable and hkBCG group ($P = 0.1$)

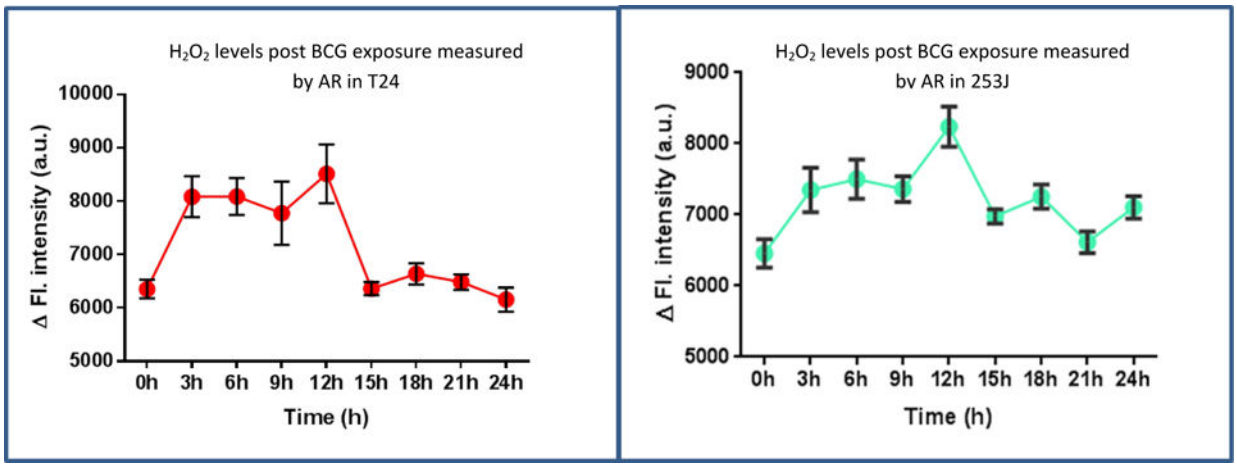


Figure 2.

Real time global profiling of ROS post BCG exposure: T24 and 253J cell lines were exposed to BCG and H_2O_2 was measured using AR at different time points post BCG exposure. Exposure of UC cells to BCG significantly increased the levels of H_2O_2 generation. The increase could be detected from 3h till 12 h ($p < 0.001$) in T24. In 253J, increase in H_2O_2 could be detected from 3h onwards till 18h post BCG exposure h ($p < 0.01$). Values on Y axis show the difference between “time correct” control and BCG treated cells.

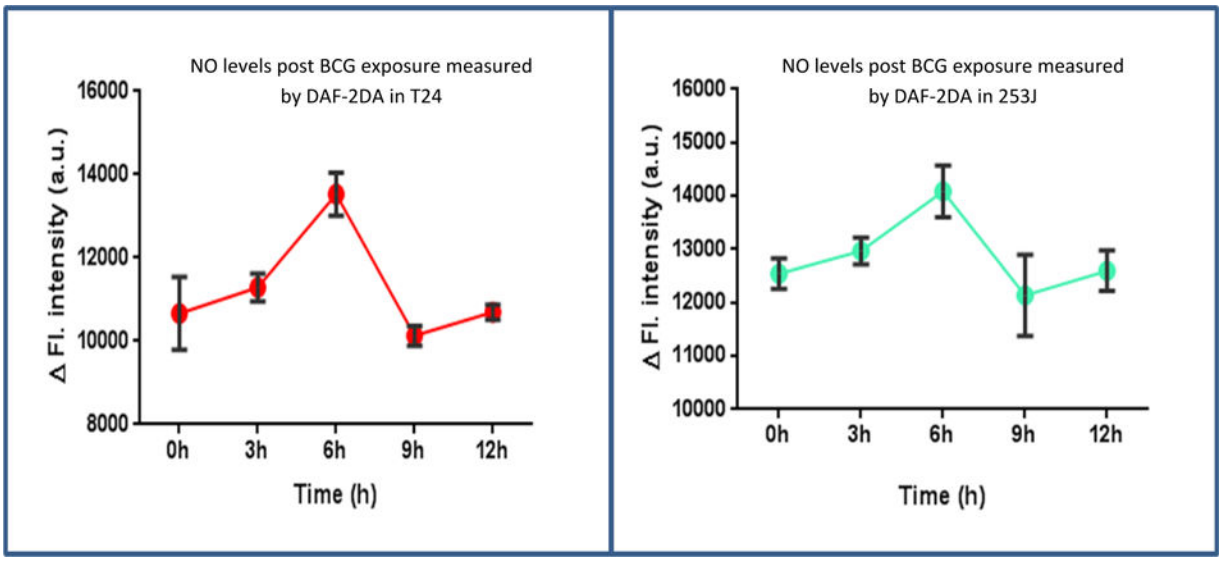


Figure 3.

Effect of BCG on real time global profiling of RNS: T24 and 253J cell lines were exposed to BCG and NO levels were measured using DAF-2DA at different time points post BCG exposure. NO levels starts peaking up at 3h and reach to significantly higher levels at 6h in both T24 ($p < 0.01$) and 253J ($p < 0.01$) cell lines compared to control. No significant difference in NO levels between control and treated group were observed 6h onwards (T24, $P = 0.57$; 253J, $p = 0.28$). Values on Y axis show the difference between “time correct” control and BCG treated cells.

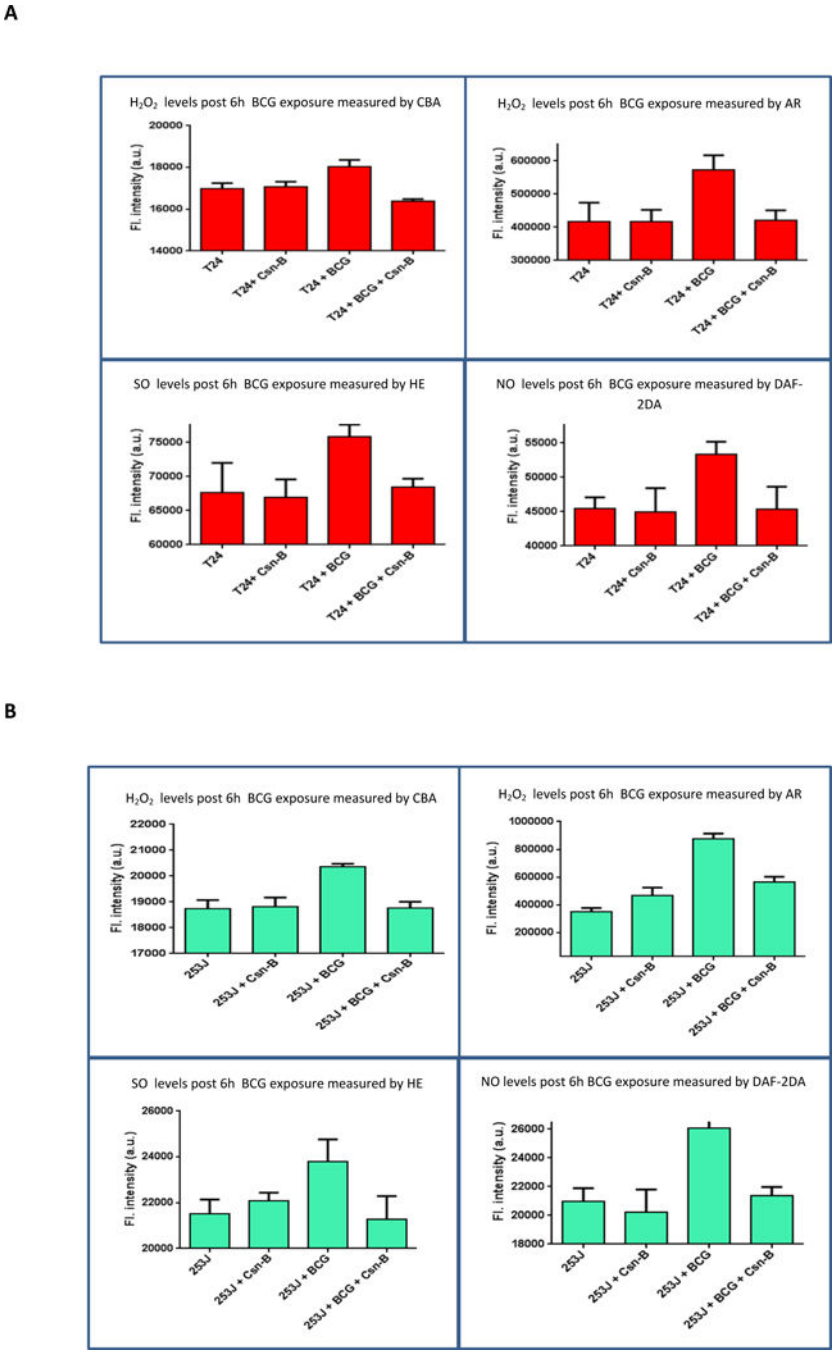


Figure 4.

Effect of BCG internalization on real time ROS/RNS profiling: T24 and 253J cells were exposed to BCG for 6h. Post BCG exposure, H_2O_2 levels were measured using CBA and AR, O_2^- levels were measured using HE and NO levels were measured using DAF-2DA. Treatment groups included Untreated, Cytochalasin-B treated, BCG treated and BCG with cytochalasin-B. H_2O_2, O_2^- and NO levels significantly decreased in the BCG with cytochalasin-b treatment group in both cell lines, relative to BCG alone. ($p < 0.05$ for H_2O_2,

$p < 0.01$ for O_2^- and $p < 0.05$ for NO). The difference in Cytochalasin-b control group and cytochalasin-b plus BCG group was statistically non-significant ($p = 0.4$).

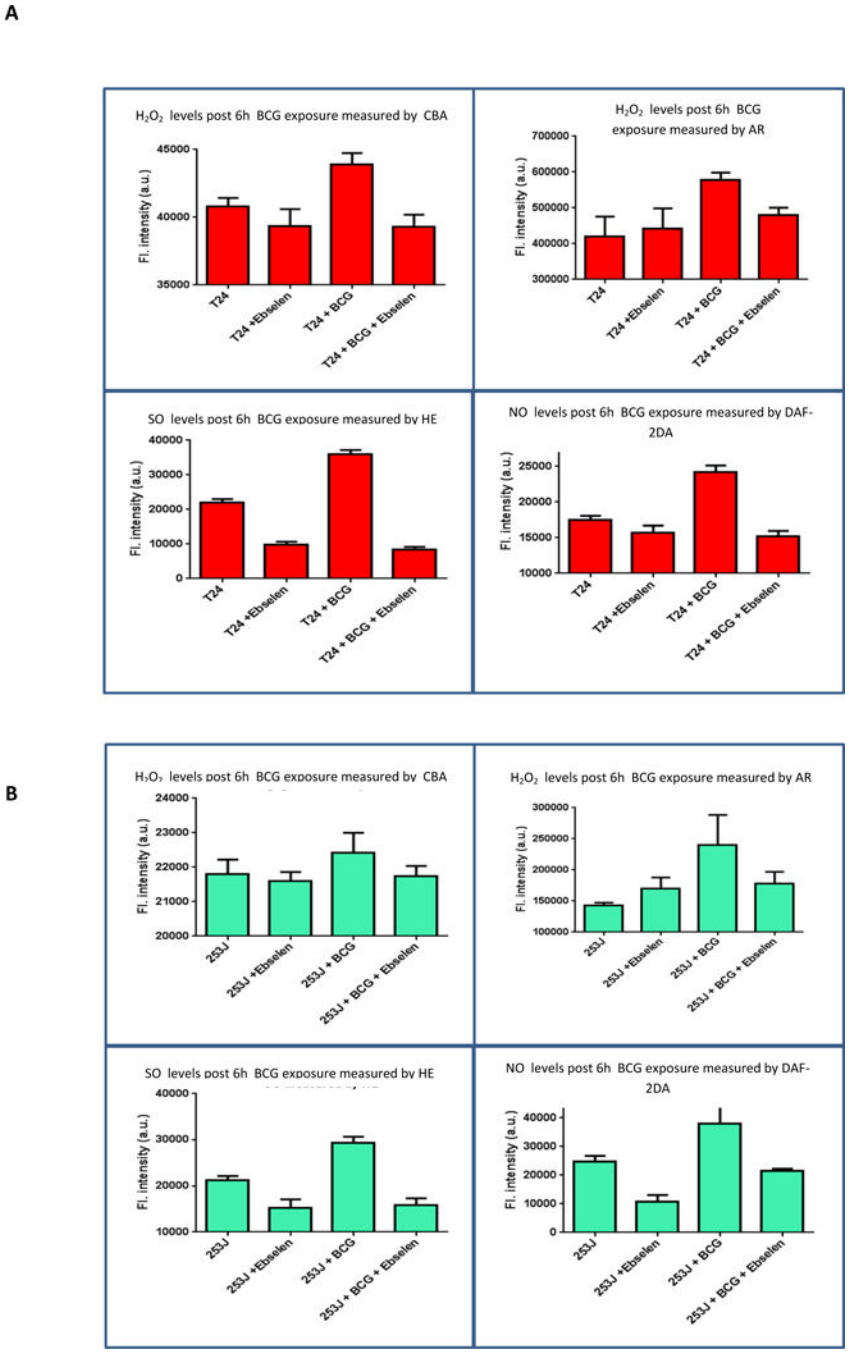


Figure 5.

Effect of ebselen on real time ROS/RNS global profiling: T24 and 253J cells were exposed to BCG for 6h. Post BCG exposure. H_2O_2 levels were measured using CBA and AR, O_2^- levels were measured using HE and NO levels were measured using DAF-2DA. Treatment groups included Untreated, ebselen treated, BCG treated and BCG with ebselen. Compared to the BCG treated group, BCG with ebselen treated group had significantly reduced levels of ROS/RNS. ($p < 0.05$ for H_2O_2, $p < 0.001$ for O_2^- and NO). The difference in ebselen control group and ebselen plus BCG group was statistically non-significant ($p = 0.7$).

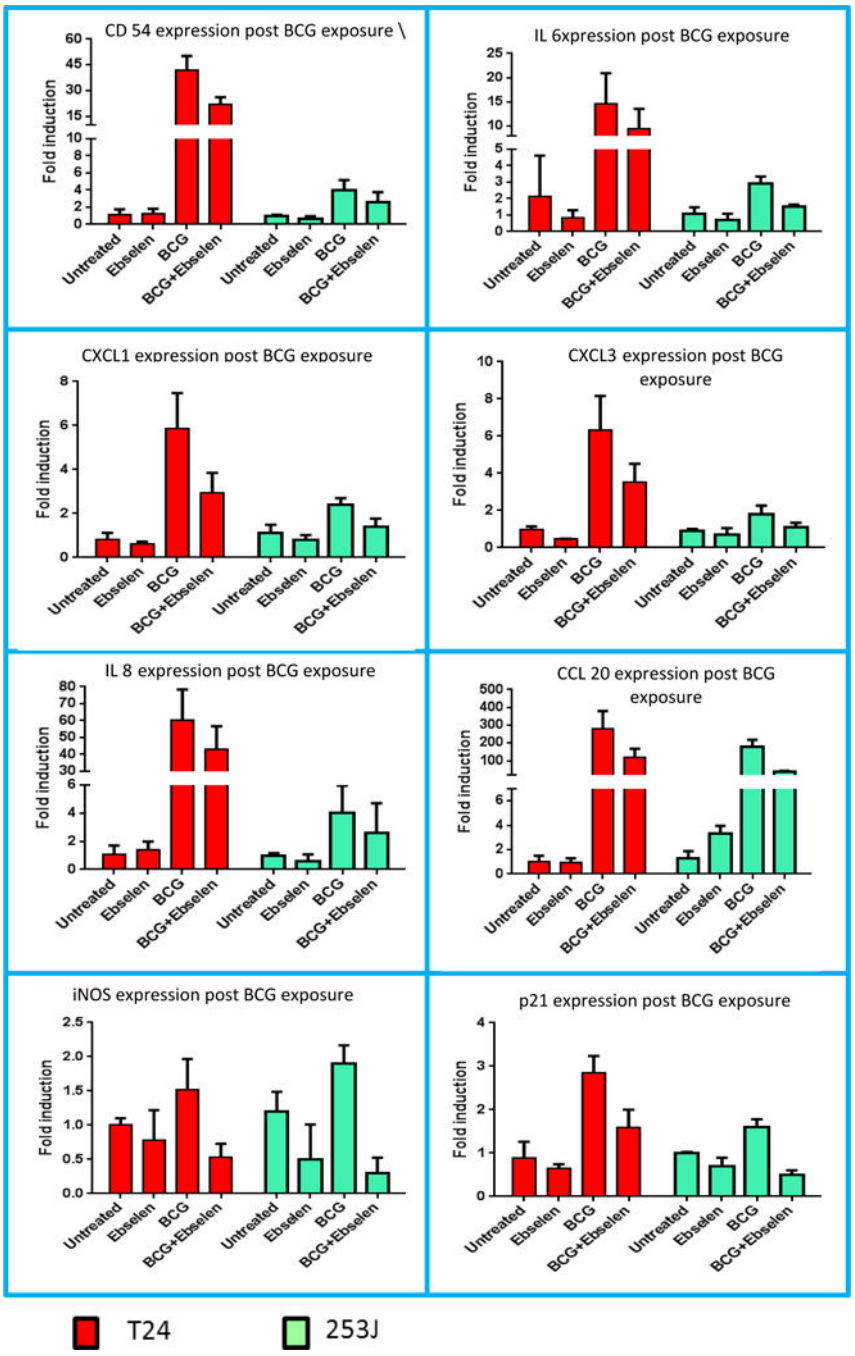
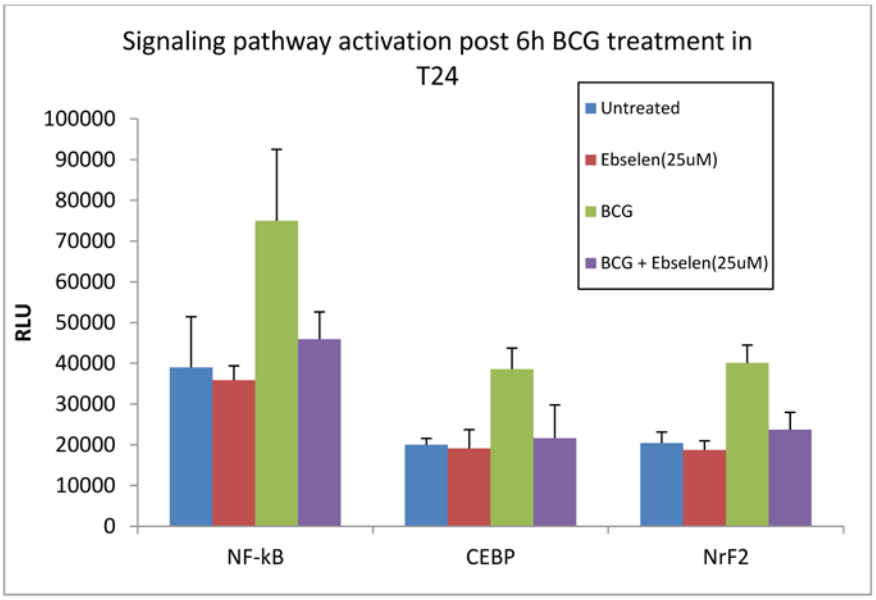


Figure 6.

Effect of free radical scavenger on Gene Expression: T24 and 253J cells were exposed to BCG. Post BCG exposure, gene expression of various genes was measured using q-RT PCR. Treatment groups included untreated, ebselen treated, BCG treated and BCG with ebselen. BCG exposure resulted in significant increased the expression of 8 immune response genes as measured by qRTPCR in both cell lines (ANOVA, $p < 0.05$). Ebselen in combination with BCG reduced UC cell expression of all measured genes with 7 of 8 and 6 of 8 reaching the level of statistical significance in 253J and T24 cells respectively (ANOVA, $P < 0.05$).

a.



b.

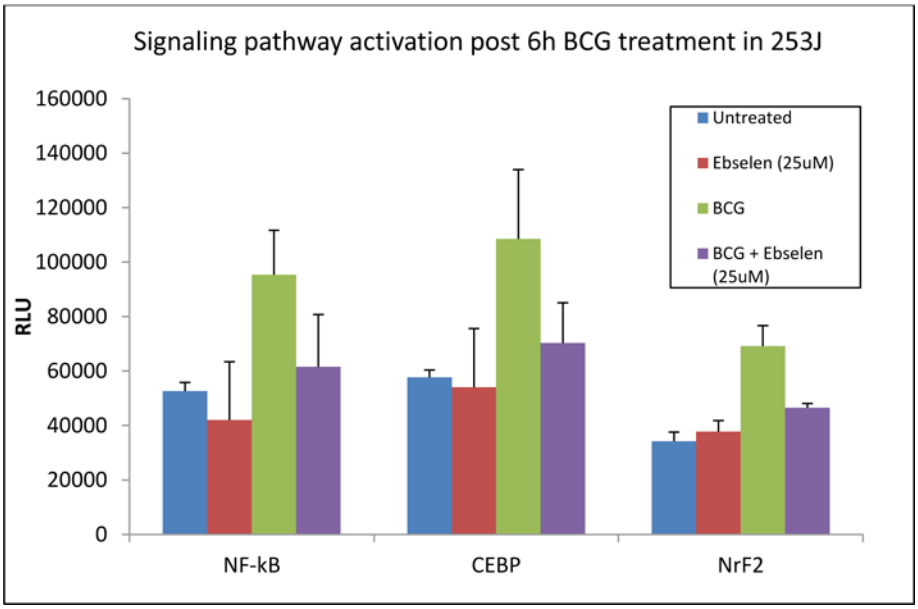
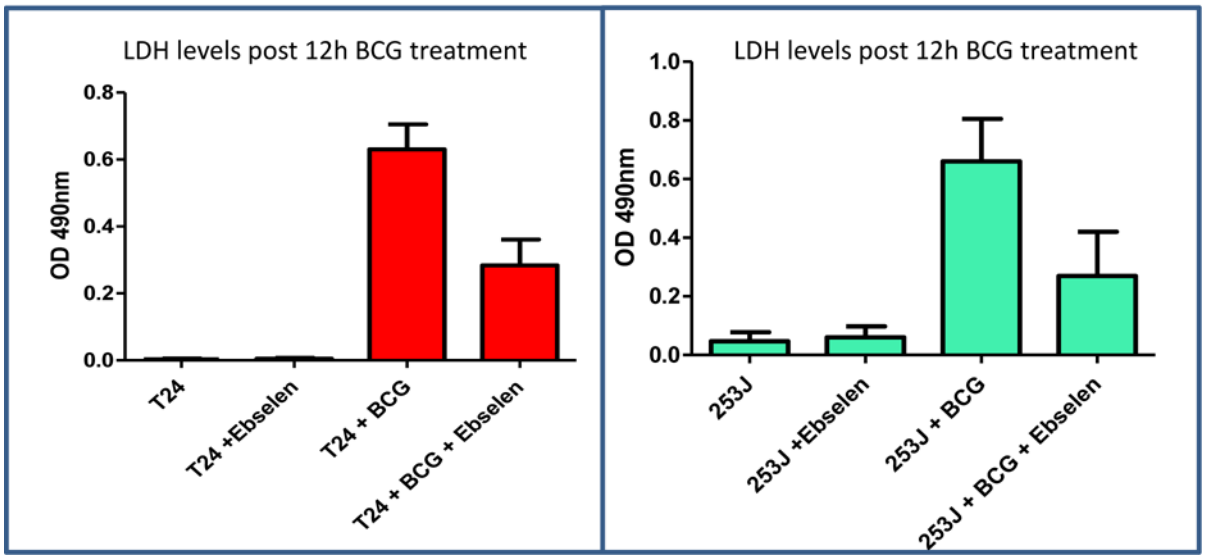


Figure 7.

Effect of ebselen on signaling pathways: T24 and 253J cells were exposed to BCG for 6h. Treatment groups included Untreated, ebselen treated, BCG treated and BCG with ebselen. BCG exposure significantly increased activation of NF-κB, CEBP and NrF2 luciferase reporter construct in both cell lines ($p < 0.01$). Ebselen in combination with BCG significantly reduced NF-κB ($p < 0.01$), CEBP ($p < 0.05$) and NrF2 ($p < 0.05$) reporter activation.

a:



b.

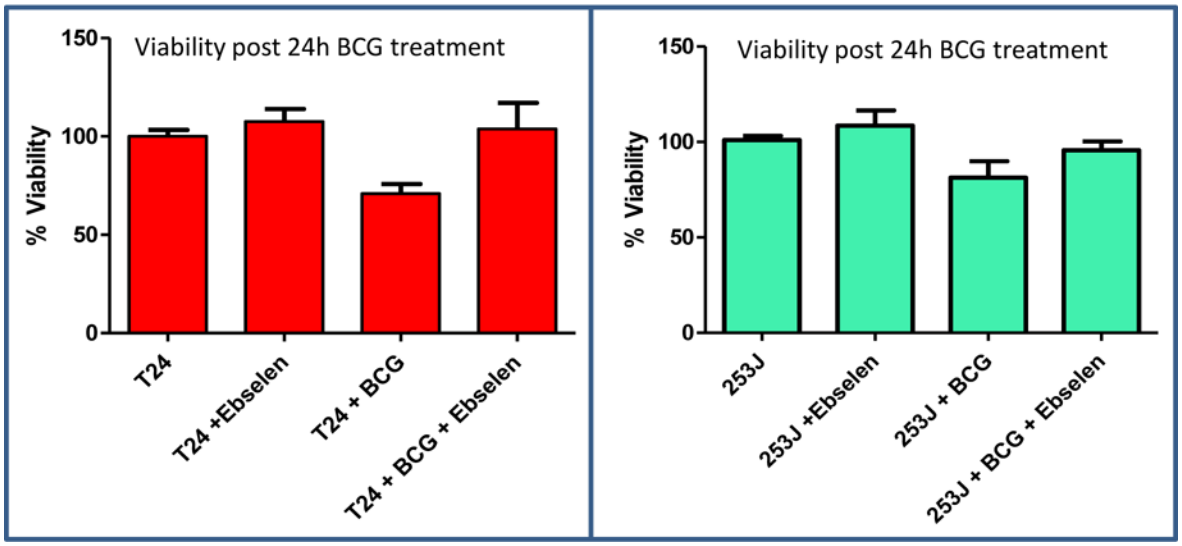


Figure 8.

a: Effect of ebselen on BCG cytotoxicity: T24 and 253J cells were exposed to BCG for 12 or 24h. Treatment groups included Untreated, ebselen treated, BCG treated and BCG with ebselen. BCG exposure significantly increased LDH release in T24 and 253J cells ($p < 0.001$). Ebselen pretreatment significantly decreased LDH release relative to BCG alone in both cell lines after 12 h (T24 $p < 0.001$; 253J $p < 0.01$). b: BCG exposure for 24 h significantly inhibited cell proliferation in 253J and T24 cells, respectively (T24 $p < 0.005$; 253J $p < 0.05$). Treatment of cells with ebselen prior to BCG treatment inhibited BCG's antiproliferative/cytotoxic effect in 253J and T24 cells ($p < 0.05$).