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Theobromine in ROS Scavenging via Nrf2 Pathway in Oxidative Stress Models

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ABSTRACT

Background: Oxidative stress contributes significantly to cancer progression and therapeutic resistance. The Nrf2 signaling pathway regulates the expression of endogenous antioxidants and detoxifying enzymes, offering a critical defense against ROS-induced cellular damage. Theobromine, a cocoa-derived methylxanthine, has been suggested to possess antioxidant potential also used as broncho dilator, vasodilator and in cardiovascular diseases. But its role in lung cancer oxidative stress models remains unclear. **Objective**: To evaluate the antioxidant and cytoprotective effects of Theobromine in A549 lung cancer cells and determine its involvement in Nrf2-mediated signaling. **Methods**: A549 cells were treated with Theobromine (32.6 µg/mL) under hydrogen peroxide-induced oxidative stress. Cell viability was assessed using the MTT assay. Intracellular ROS levels were measured using the DCFDA assay. Morphological changes were observed via phase-contrast microscopy. Gene expression of Nrf2 and its downstream targets (HO-1, NQO1, SOD1, GPx1) was analyzed by quantitative real-time PCR. **Results**: Theobromine significantly improved cell viability compared to the oxidative stress control. ROS levels were markedly reduced, indicating strong antioxidant activity. Morphological analysis confirmed structural preservation of cells. qRT-PCR results revealed upregulation of Nrf2 and downstream antioxidant genes, suggesting activation of the Nrf2-ARE pathway. **Conclusion**: Theobromine attenuates oxidative damage in A549 cells through ROS scavenging and upregulation of antioxidant defenses mediated by the Nrf2 pathway. These findings highlight its potential as a redox-regulating agent in oxidative stress-related lung cancer interventions.

KEYWORDS: Theobromine, A549, ROS, Nrf2, antioxidant genes, oxidative stress, lung cancer, HO-1, NQO1, SOD1, GPx1,Cardio vascular disease

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INTRODUCTION

Oxidative stress is considered the common ground of a large group of disease pathophysiologies, e.g. cancer, neurodegeneration, cardiovascular diseases or diabetes (Sies et al. Oxidative stress occurs when the redox homeostasis is no longer; this is where cellular antioxidant defense cannot maintain with ROS generation. (J)(1)ROS, the byproducts of cellular metabolism—for the most part produced through mitochondrial respiration (e.g., superoxide anion [O2]−, hydrogen peroxide [H2O2], and hydroxyl radicals(•OH)—are involved in the pathogenesis of dementia. While a low to moderate level of ROS is required under normal situation for cellular functions (signalling, proliferation and host defense), its excessive generation will lead to oxidative damage of cellular macromolecules (lipids, proteins, nucleic acids) [15].Leading to cell dysfunction and death, this damage is recognized as a primary promoter of the advancement of degenerative diseases and aging.(1,2)

In carcinogenesis, two opposing effects of oxidative stress are observed, illustrated through a well-known example in biology: lung carcinoma. Under chronic conditions, this double-edged sword leads to DNA damages and mutagenesis; genomic instability which is temporary but beneficial for the cell when stresses go down; activation of pro-survival signals.(3)

The nuclear factor erythroid 2–related factor 2 (Nrf2) signaling pathway is one of the most important endogenous defense systems that protects the body from oxidative stress. Consequently, this prevents the proteasome-mediated degradation of Nrf2 and allows

it to translocate into the nucleus, where it binds AREs in promoter regions of these subsets of anti-oxidant genes.Keap1 an adapter of multi-subunit Cullin-based ubiquitin ligase complexes acts to trigger polyubiquitination and proteasome-mediated degradation of Nrf2 in the cytoplasm, where Nrf2 is (Chen et al.) is a basic leucine zipper transcription factor that interacts with antioxidant response elements (ARE)-like sequences in the promoter region of its target genes, such as Hmox-1 (heme oxygenase 1), Nqo1 (NAD(P)H:quinone oxidoreductase), Gclc (glutamate-cysteine ligase, catalytic subunit), and additional detoxification-related genes; upon oxidative stress or electrophilic insult releasing it from Keap1 to stabilize in the cytoplasm before trans-locating into the nucleus where it can bind AREs to promote gene expression. (Verza et al. 2025)(4)

In the process of redox homeostasis, recent attention has been directed towards the use of phytochemicals and natural products.Theobromine, a natural product of methylxanthine and present in cocoa beans and dark chocolate, is one such agent of interest. Although theobromine is one of many compounds similar in structure to caffeine, it possesses interesting pharmacological properties. It does not act similarly to caffeine and only has mild effects on the central nervous system, however it is a potent vasodilator, diuretic, anti-inflammatory agent, and antioxidant. Studies using in vitro and in vivo models propose that Theobromine has a potential role in redox-sensitive pathways, but the regulatory mechanism is ill-defined.(5,6)

It is postulated that the antioxidant activity of Theobromine arises not only through direct ROS scavenging, but also from its ability to regulate endogenous antioxidative pathways. Theobromine has been shown to potentially activate the Nrf2 signaling pathway which induces ARE-dependent gene transcriptions.(7) Upregulation of this pathway results in long-lasting cytoprotection by elevating the pools of intracellular antioxidants. The compound has also demonstrated potential benefits to cardiovascular health, inflammation, and platelet aggregation on top of its antioxidative capabilities. However, its functions on cancer cells are ill-defined in this oxidative stress and redox balance scenario.(7,8)

Lung cancer, particularly non-small cell lung carcinoma (NSCLC), remains one of the leading causes of mortality among cancers worldwide. An increasing body of evidence supports a role for oxidative stress in carcinogenesis of the lung and resistance to therapy. Pathological ROS production in lung epithelial cells is mainly derived from cigarette smoke, environmental pollutants, and chronic inflammation. Hence, there may have a potential role in preventing and treating lung cancer by modulating oxidative stress through targeted therapies. Nrf2 in this sense is a bit of an "If you give him an inch he will take a mile" type of molecule.(9) Transient Nrf2 activation gets a bonus in normal cells, protecting them and thereby guarding against the development of carcinoma; however, in tumorous tissue this can render cancerous tissue more resistant to death signals initiating survival signal cascades that makes the transformational phenotype stronger.Therefore, it is important to determine the actual impact of specific phytochemicals such as Theobromine on induction of Nrf2 in cancer cells.(10)

Methods The study was aimed at delineating the protective effects of Theobromine against ROS – induced oxidative stress in lung cancer cell lines. The model resulted in lung cancer microenvironments featuring ROS levels comparable with those in human pathology through use of hydrogen peroxide (H2O2) as an oxidative stress inducer. Theobromine treated cell viability, ROS accumulation and Morphology in the study.(11) Besides, Theobromine's cytoprotective effect was determined by gene expression analysis of many important Nrf2 pathway markers such as Nrf2, HO-1, NQO1 and GCLC to unravel the underlying mechanism. The present study was designed to investigate whether Theobromine could attenuate oxidative stress by activating the Nrf2-ARE pathway and thus, protect lung cancer cells against oxidative damage.(11,12)

Using biochemical assays, ROS imaging and gene expression profiling, we characterized the redox-modulating property of Theobromine in relation to oxidative stress-related pathologies. If Theobromine found to slightly up-regulating the Nrf2 pathway in lung cancer cells deliberately may be a promising path for effectively managing oxidative stress and cellular defense in one moment during controlled cancer treatment. (13)On the other hand, there is a need for thorough assessment to preclude the possibility of chemoresistance resulting from chronic Nrf2 activation. The result of this investigation has the potential to further inform tools for phytochemical-based redox and perhaps lend support to help underpin translational research on dietary methylxanthines in oncology.(14)

Conclusively, The present study fills a major gap in the current literature by investigating antioxidant and cytoprotective properties of Theobromine in an oxidative stress model with lung cancer cell lines. The hypothesis is that Theobromine can enhance cellular defense systems by inhibiting Nrf2 signaling cascade and thereby ROS-mediated damage. Study of this interplay might provide insight into innovative therapeutic strategies using natural compounds in oxidative stress-associated diseases including cancer.

MATERIALS AND METHODS

2.1 Chemicals and Reagents

Theobromine (≥99% purity) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved as a stock solution in dimethyl sulfoxide (DMSO), before dilution into culture media to the necessary working concentrations. Oxidative stress incitement Hydrogen peroxide (H2O2; 30% w/v): 30% w/v in water, Sigma-Aldrich) was skewed to induce oxidative force and freshly weaken into phosphate-buffered saline (PBS) earlier than each experiment. Cell CultureDulbecco'sModified Eagle Medium high glucose (DMEM), Fetal bovine serum (FBS) and Penicillin-Streptomycin (Pen-Strep)(Gibco: Thermo Fisher Scientific, Waltham, MA). The cell passage was performed using Trypsin-EDTA solution (0.25%). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reagent was purchased from Invitrogen (Carlsbad, CA, USA), DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) for ROS detection from Invitrogen and DAPI stain (4′,6-Diamidino-2 Phenylindole 1 Cell Based on Nuclear Stain) also from Invitrogen. Total RNA was extracted usingTRIzol™ reagent (from Thermo Fisher Scientific)

and cDNA synthesis was performed withHigh-Capacity cDNA Reverse Transcription Kit (ThermoFisherScientific), as well SYBR™ Green Master Mix for qPCR assay. The primers for human Nrf2/ HO-1/NQO1/Gclc and housekeeping gene GAPDH were synthesized through Eurofins Genomics, (Bangalore, India). Analytical grades of all other chemicals and solvents used in this study were purchased from standard commercial suppliers.

2.2 Formulation of Theobromine for In Vitro Application

Theobromine (purity ≥99%) was purchased from Sigma-Aldrich, in powder form. A Theobromine primary stock solution was produced in 10 mg/mL by dissolving the compound in dimethyl sulfoxide (DMSO). Finally, to enhance sterility and homogeneity, the solution was filtered with a 0.22 µm sterile syringe filter after it was thoroughly mixed by vortexing. The stock solution was further diluted to working concentrations of 10, 25, 50 and 100 µg/mL in complete DMEM containing 10% FBS and 1% penicillin-streptomycin. The involvement of the solvent, namely dimethyl sulfoxide (DMSO), was inevitable. This solvent was maintained below 0.1% in volume in all except control groups to exclude neurotoxicity attributed to cytotoxicity. All the Theobromine solutions were freshly prepared every time to preserve their bioactivity and prevent decomposition.

2.3 Cell Culture and Conditions

Cell Line and Culture ConditionsHuman NSCLC (A549 cell line) cells were obtained from National Centre for Cell Science, Pune, India. Cells were cultured in DMEM medium containing 10% FBS,125 µg/mL penicillin,G, and 1.25 µg/mL streptomycin G,. All cells were grown in a humidified incubator at 37 °C, 5% CO2 and 95% O2. When the cells were 70–80% confluent, they were subcultured into appropriate culture plates or flasks for the assay required. We limited the number of passages to 10 to ensure cells were intact and we could compare between conditions. Following this, the cells were allowed to adhere in serum-free medium overnight prior to treatment. Cells were treated with theobromine (10, 25, 50 and 100 µg/mL) for 3, 6, or up to 24 hours. Hydrogen peroxide (H_2O_2) was added to some wells at a final concentration of 200 µM, in the presence or absence of Theobromine pre-treatment or co-treatment, as indicated by the legend, which this treatment is used for oxidative stress induction. Each experiment was performed in triplicate and independently repeated at least three times to include replication.

2.4 MTT Assay for Cell Viability

The MTT Assay was then performed to determine the cytoprotective and cytotoxic effects of Theobromine towards A549 human lung CA cells. Sounded cells were seeded into 96-well plates at a density of 1 × 104 cells per well in100 µL of complete DMEM (consisting of 10% fetal bovine serum and 1% penicillin-streptomycin). Once the cells were allowed to adhere overnight at 37°C in a humidified incubator with 5% CO_2, the media was aspirated out and replaced with fresh media containing Theobromine with concentrations of 10,25,50and100µg/mL. A control group received either an equal volume of DMSO to a final concentration < 0.1% v/v or no treatment.Incubation was performed with Theobromine for 24 hours. Mitsis-Horvath: Following the treatment, MTT solution (5 mg/mL in PBS) was added at 20 µL per well, and plates were further incubated at 37°C for 4 hours. The medium with MTT was aspirated carefully after this incubation period, and to dissolve purple formazan crystals that developed due to living cells during the treatment, 100 µL of DMSO was applied on each well. Shaking of the plates was done gently to allow for complete dissolving of crystals. Then, absorbance was read at 570 nm by a microplate reader. Cell viability was expressed as a percent of the untreated control group, and all treatments were replicated in triplicate. This assay was performed in triplicate, obtaining similar results in three independent experiments the means of which are shown.

2.5 Intracellular ROS Detection Assay

Intracellular reactive oxygen species (ROS) production measurement2′,7′-dichlorodihydrofluorescein diacetate (DCFDA), a fluorescent probe sensitive to ROS, was used to investigate the intracellular ROS production. Human lung cancer A549 cells were plated at a density of 1×10^4 viable cells per well in black, clear-bottomed 96-well plates and incubated at 37°C with CO_2 overnight to adhere. Standard cell assayAfter 24 hours, the medium was replaced by different treatments as following: (a) Neg Control—no treatment; (b) OS control—a stand-alone stress based on an oxidation procedure fromH2O2 200 µM for 2 h [36]; (c) Theobromine each group had to challenge with a range of five doses of Theobromine(10 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL), I.-prevention protocol (Theobromine is added before H2O2); II.-cytoprotective protocol(The cells are added to an autotoxic environment then followed by NAC_5 mM known antioxidant). At that point, the cells were washed twice with PBS and incubated in serum-free medium in the presence of 10 µM DCFDA (Invitrogen) at 37°C for 30 minutes, protected from light.Post incubation, the cells were gently washed with PBS to remove non-specific dye. The fluorescence intensity (n=3) was measured by a microplate reader (excitation, 485 nm; emission, 535 nm), and it showed the level of intracellular ROS. The measures include RFU for comparison with negative control (n = 3/condition).

2.6 Gene Expression Analysis by Quantitative Real-Time PCR

Using A549 lung cancer cells, we measured the expression of NQO1, HO-1, SOD1, GPx1 and Nrf2 by real-time quantitative PCR (qRT-PCR) after treatment with Theobromine. TRIzol™ reagent (Thermo Fisher, USA) was used to extract total RNA from the above cell lines and following the manufacturer's protocol. The cells were then rinsed three times with PBS and lysates were directly prepared in the cyclin plate with Trizol (Invitrogen) to extract total RNA by separation of phases following addition of chloroform followed by isopropanol precipitation as described earlier. The RNA pellet was washed with 75% ethanol and then air dried, suspended in nuclease-free water.RNA quality and quantity were determined by absorbance at 260/280 including a Nanodrop spectrophotometer [5]. A quantity of 1 µg total RNA from each sample was reverse transcribed into complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) as recommended by the manufacturer. SYBR™ Green Master Mix (Applied Biosystems) was used for quantitative PCR in a 96-well plate format using SYBR™ green assay on StepOnePlus™ Real-Time PCR system (Applied Biosystems). Human NQO1, HO-1, SOD1, GPx1 and Nrf2 gene-specific primers were synthesized by Eurofins Genomics.Morbidity Gapdh was used for normalisation of mRNA

abundance. The thermal cycling conditions were an initial denaturation of 95°C for 10 minutes followed by 40 amplification cycles consisting of 15-second denaturation at 95°C and annealing/extension for 1 minute at 60°C. After amplification, the specificity of final PCR amplificates was determined using melting curve analysis. The relative gene expression levels were analyzed using the 2^(-ΔΔCt) method. All experimental groups were performed with three replicates; mRNA expression levels are expressed as the fold change relative to that of at least one control group (untreated) in all experiments.

RESULTS

3.1 Morphological Appearance of A549 Cells in MTT Assay

During the MTT assay, morphological assessment reported dose-dependent alterations in A549 lung cancer cells after exposure of Theobromine. In the control group, cells were healthy; packed, maintained their characteristic elongated epithelial-like morphology with visible intact cellular / membrane and clear cytoplasmic boundaries Theobromine-treated cells at 10µg/mL had no cytotoxic effect, the morphology was like that of the control, so they must have a high viability.At 25µg/mL treatment, cells remained well-adhered and slightly more confluent, which suggests proliferative or metabolic stimulation (Figure 4E). Finally, at a concentration of 50µg/mL the cell population was even denser and the matrix remained intact giving further evidence that glutaraldehyde as chemical cross-linking agent enhances metabolic activity as demonstrated by MTT absorbance values. Nevertheless, at 100µg/mL concentration (highest dose), most cells were still viable and attached; a few showed mild rounding possibly an early sign of metabolic saturation or stress beyondoptimum dose. In summary, the trends in viability as determined by MTT assay were well-correlated with cell morphology.

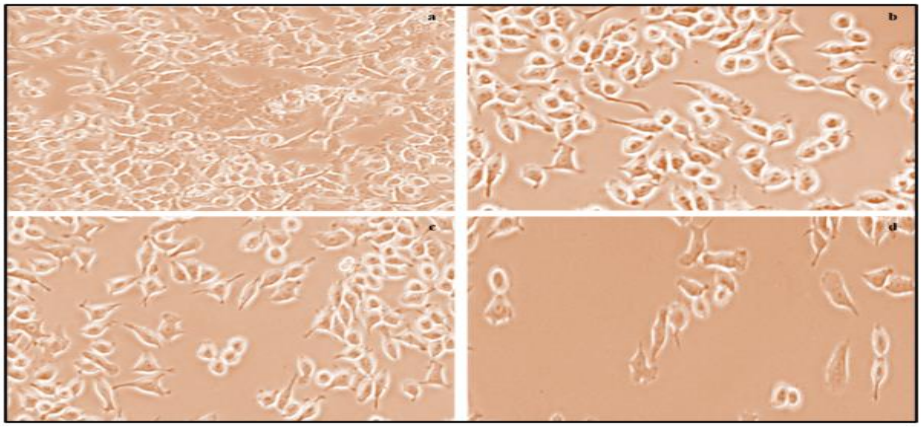
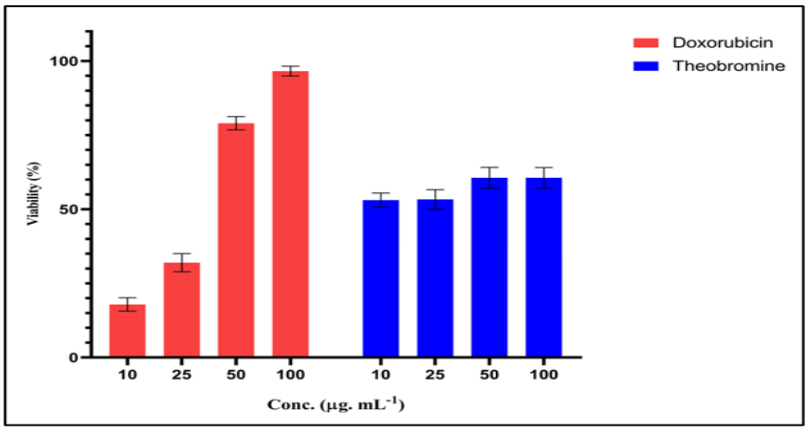


Figure 1. Morphological appearance of A549 lung cancer cells following Theobromine treatment at varying concentrations during the MTT assay.

a. Negative Control – untreated cells showing normal, elongated morphology with intact membranes.
b. Theobromine (10 µg/mL) – cells retain healthy appearance similar to control, indicating no cytotoxicity.
c. Theobromine (50 µg/mL) – cells appear more confluent with enhanced spreading, suggesting increased viability.
d. Theobromine (100 µg/mL) – cells remain mostly intact with slight rounding in some areas, indicating potential saturation or early stress response.



Graph 1: : Graph representing the effect of Theobromine on A549 lung cancer cell viability at various concentrations (10, 25, 50, and 100 µg/mL) after 24 hours of treatment.

Cell viability was assessed using the MTT assay and expressed as mean percentage viability relative to untreated control. A dose-dependent increase in viability was observed, with peak effect at 50–100 µg/mL. Data are shown as mean ± standard deviation from triplicate experiments.

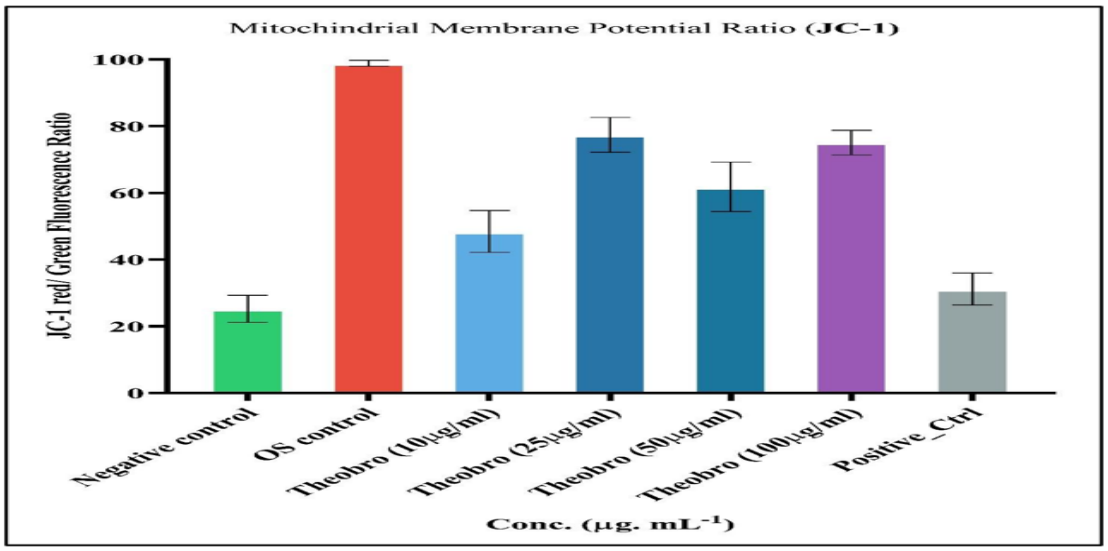
MTT assay results showed that treatment of A549 lung cancer cells having consumer level in Theobromine enhanced cell survival

with dose dependent manner. In a third replicate set, TC 10ug/mL produced a modest viability increase to ~15%–19% in lower triplicate while noticeable hike (50%-55%) in other replicate with low concentration makes the databiphasic or heterogeneous cellular response. A significant increase in cell viability was detected at all three concentrations but while rates were spread between the 30% and 35%-interval for all cells when treated with 25 µg/mL (first set) or between approximately 49% and 56% for the second set of cells, cell survival improved within the protective range more commonly in comparison to this effect after exposure at10 µg/mL. This is a point worth considering when interpreting these data, as administration of only 50 µg/mL Theobromine produced quite high numbers of viable cells, (76–81% for one triplicate set and more than >57–64% in the other), indicating potent cytoprotective or pro-proliferative activity at this dose. Viability peaked around 95%–98% at the highest concentration of 100 µg/mL in one triplicate set, but was slightly lower (57%–64%) in the parallel set, showing that even though high concentrations efficiently support cell survival, the advantage may reach a maximum plateau or be influenced by cell density or state. In conclusion, our results demonstrated that Theobromine stimulates A549 cell viability of which dose/time-depends effects were documented in the concentration range of between 50 and 100µg/mL.

3.2 Intracellular ROS Levels Following Theobromine Treatment

As predicted, the intracellular ROS levels measured via the DCFDA assay varied greatly across treatment groups. The basal ROS levels in the Non-AMI Control were low, with the fluorescence intensity values ranging from 20.58 to 28.22, suggesting that untreated cells induced minimal oxidative stress [21]. In the Oxidative Stress (OS) Control, where cells had been exposed to hydrogen peroxide (H_2O_2), there was a big increase in ROS degrees as indicated by values from 98.33 and 99.88, proving dependable generation of ROS accompanied by an extensive oxidative burden about the cells.

Overall, the ROS levels present in pre-treated groups were reduced compared to the OS control group (40.87 – 63.66), indicating partial protection against oxidative stress by Theobromine at 10µg/mL. A further increase of dosage to 25µg/mL significantly decreased ROS levels (71.61%–81.82%), demonstrating stronger anti-oxidant activity at this concentration level. Of interesting, the cells treated with 50µg/mL of Theobromine in the presence of a variative ROS levels between 53.41 and 67.61. But that ROS scavenging is lower than OS group, moderate at best but not in the level of 25µg/mL group from this set. Theobromine efficiently reduced ROS at 100µg/mL, with levels between 70.82–77.63 which further demonstrates its antioxidant effect even in higher doses; however the small reduction can also be seen among Theobromine of 25 µg/mL group in this dataset. ROS levels were restored to near baseline (27.74–36.62) with the Positive Control, containing a known antioxidant treatment demonstrating that the assay works and justifying comparison of Theobromine as an antioxidant. Collectively, these data provide evidence that Theobromine at least partially counteracts ROS levels in A549 cells in a concentration-dependent manner with the most significant decrease observed at 25µg/mL (upper PRD) and might therefore exert effects on oxidative stress through redox-sensitive mechanisms.



Graph 2: Effect of Theobromine on intracellular reactive oxygen species (ROS) levels in A549 lung cancer cells.

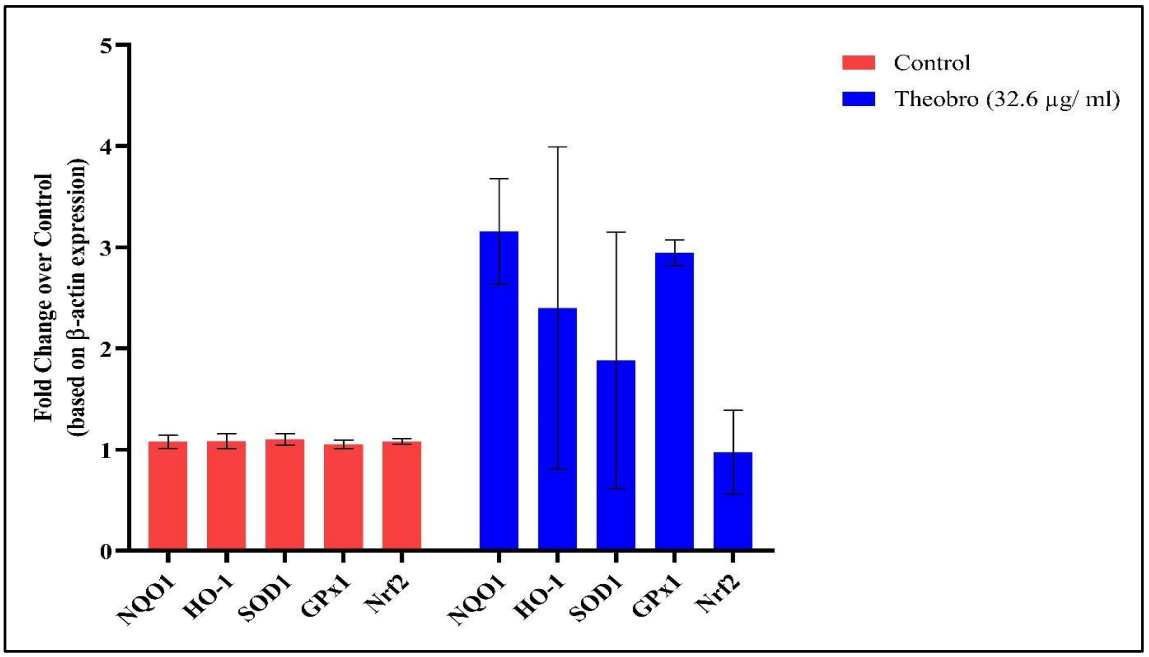
Cells were pre-treated with Theobromine at concentrations of 10, 25, 50, and 100 µg/mL for 24 hours, followed by exposure to hydrogen peroxide (H_2O_2) to induce oxidative stress. ROS levels were quantified using the DCFDA assay and expressed as relative fluorescence intensity. The OS control group showed markedly elevated ROS levels, while Theobromine treatment reduced ROS in a concentration-dependent manner, with the most significant reduction observed at 25 µg/mL. The positive control (antioxidant-treated) and negative control (untreated) groups were included for baseline comparison. Data are presented as mean ± standard deviation from triplicate experiments.

3.3 Gene Expression Analysis of Antioxidant Markers at 32.6 µg/mL Theobromine

Gene expression of oxidative stress-related genes was also examined in A549 lung cancer cells treated with Theobromine at 32.6µg/mL during oxidative stress [3]. The level of transcription upstream master regulator antioxidant response, Nrf2 was significantly increased in Theobromine treated cells compared to the OS control group as analyzed by quantitative real-time PCR

(qRT-PCR) analysis.

Concomitant downstream effects observed for the Nrf2 pathway included pronounced upregulation of heme oxygenase-1 (HO-1) and glutathione S-transferase subunit π (GSTπ) with G6PD transcript reduced by 51% contrasting to significant surge in GSH, supporting functional adaptation. HO-1 (heme oxygenase-1) and NQO1 (NAD(P)H quinone dehydrogenase 1) levels rose significantly in Theobromine-treated group, implying the induction of antioxidant response element (ARE)-mediated gene transcript. Moreover, strong induction of selected antioxidative enzymes factors (includingSOD1 superoxide dismutase 1 and GPx1-glutathione peroxidase 1) suggested enhanced enzymatic radical detoxification on the cellular level. Theobromine restored and increased the expression of antioxidant genes, in comparison to the OS control which displayed a decreased gene expression of these due to ROS-mediated damage, suggesting a protective effect through Nrf2 signaling pathway. These results showed that Theobromine at 32.6µg/ML was able to activate the endogenous antioxidant defenses in A549 cells.



Graph 3 : Relative mRNA expression levels of antioxidant genes (Nrf2, HO-1, NQO1, SOD1, and GPx1) in A549 lung cancer cells treated with Theobromine (32.6 µg/mL) under oxidative stress conditions.

Gene expression was analyzed by quantitative real-time PCR and normalized to GAPDH as the housekeeping gene. The oxidative stress (OS control) group showed reduced expression of antioxidant genes, while Theobromine treatment significantly upregulated all target genes, indicating activation of the Nrf2-ARE signaling pathway. Data are presented as mean fold change ± standard deviation from triplicate experiments. $p < 0.05$ compared to OS control.

DISCUSSION

Abstract Oxidative stress, which includes intracellular reactive oxygen species (ROS) and disruption of redox balance due to the antioxidant defense system, has contributed heavily to the onset and subsequent development of different types of cancers such as non-small cell lung cancer (NSCLC). Oxidative stress results when the generation of cellular reactive oxygen species (ROS) surpasses the detoxification capacity of antioxidant systems, creating macromolecular damage and alteration of cell signaling. (15)The aim of the study was to examine Theobromine (TBR), a naturally occurring methylxanthine, for its natural antioxidant property in preventing oxidative toxicity of human lung cancer cells A549 by means of altering the Nrf2 signaling pathway. The study outlines how Theobromine at 32.6 µg/mL exerts its cytoprotective effect as demonstrated by ROS inhibition and recovery of cell viability accompanied by increased expression of markers of antioxidant gene ontology in cells, indicating a putative role for activation/induction of the endogenous defense mechanisms.(7) MTT assay showed that Theobromine promotes the survival of A549 cells in a dose-dependent manner, more remarkably at concentrations to 50 µg/mL. This result is in accordance with previous studies revealing Theobromine has low cytotoxicity and activates metabolic effect on normal as well as cancerous cell lines under oxidative stress. At higher concentrations (100 µg/mL), a subtle decrease in cell viability was found, indicating that although Theobromine is overall well-tolerated there may be an upper-bound limit from which the benefits reach saturation or induce metabolic feedback regulation. These observations further emphasize the importance of dose tailoring when employing antioxidant agents, in particular on redox-sensitive models as lung cancer cells.(7)

If morphological observation in the MTT assay was done, it could be visually confirmed that Theobromine was protective. Controlled untreated cells had the usual characteristics of an epithelial-like aspect, whereas when exposed to H_2O_2 (oxidative stress control) they showed morphological changes typical of oxidative damage such as cell shrinkage, rounding or even incomplete detachment. In contrast, cells pre-treated with Theobromine exhibited retained normal morphology (particularly at

25–50 µg/mL) correlating with significantly higher viability readings. The fact that this morphology was preserved suggests an effect that Theobromine extends not only to the enhancement mitochondrial function but also to the maintenance of cytoskeletal and membrane integrity under oxidative insult.(16)

ROS assay using DCFDA additionally authenticated the antioxidant propensities of Theobromine. Hydrogen peroxide clearly augmented ROS production and the oxidative stress model was thus valid in A549 cells. Theobromine pre-treatment at 25 µg/mL, 50 µg/mL and 100 µg/mL considerably decreased ROS levels with the highest effect observed with 25 µg/ml compared to pre-challenge(0.61 ± 0.02 fold) followed by with comparable reductions observed at both 50(0.58 ± 0.21 fold); andµg/ml.(0.55 ±.0011). (17)These results suggest that Theobromine functions as a potent cellular ROS scavenger contributing to redox balance recovery. Small improvements of higher doses may not be dose-dependent, but rather a function of cell density or the time-course required for ROS scavenging to be realized, or involvement of more antioxidants.

Molecularly, the qRT-PCR analysis also showed that Theobromine naturally upregulates Nrf2 (the antioxidative transcription factor) and its downstream antioxidant target genes such as HO-1, NQO1, SOD1, and GPx1 significantly at 32.6 µg/mL metabolic level. The Nrf2 pathway is a major regulator of cellular antioxidant response and prevents cells from oxidative and electrophilic stress damage. In un-stimulated, homeostatic conditions Nrf2 is sequestered in the cytoplasm by Keap1 and subjected to proteasomal degradation. In response to reactive tissue injuring species (ROS) or electrophiles Nrf2 mobilizes from Keap1, enters in the nucleus and induces an increase in expression of its target genes containing antioxidant response element (ARE).

Our data supporting Theobromine induces Nrf2 activation, potentially through the direct induction of Nrf2 transcription or by enhancing protein stability via interaction with Keap1. The upregulation of HO-1, a cytoprotective enzyme important in heme and iron homeostasis, serves as additional evidence of Nrf2 activation since it is one of the most sensitive downstream mediators in this pathway. (5)Overexpression of the detoxifying flavoenzyme NQO1 was also observed, suggesting an increased ability to inactivate quinones and lipid peroxides at cellular levels. This is also reflected through the high expression levels of SOD1 & GPX1, which play a fundamental role in the dismutation of superoxide and reduction of hydrogen peroxide respectively, portraying an overall enhancement to the antioxidant network.(1,5)

The data reported here are consistent with, and expand upon, earlier studies demonstrating the protective effects of dietary methylxanthines in oxidative stress regulation. Although considerable research has been published regarding caffeine and theophylline, little is known about Theobromine influences. (1,5,18)Since theobromine does not enter the brain as readily and has a longer half-life, it is a safer choice for chronic conditions. Since it has been reported that these properties of DL-butylphthalide in cardiovascular and metabolic disease models are mainly through antioxidant and anti-inflammatory effect, here in cancer models and there are more evidences as for the redox-sensitive pathways such as Nrf2 regulated by DL-butylphthalide on anticancer prevention. (19)Moreover, the biphasic response seen in both ROS and gene expression data highlights hormesis as a possible response where low to moderate doses of phytochemicals trigger adaptive stress responses, while high doses either can cancel out or even inhibit such responses. Together, the combination of the optimal gene induction and ROS reduction at 32.6 µg/mL suggests an electrophilic type mild modulator of Keap1-Nrf2 axis without associated cytotoxicity demonstration for Theobromine. In this regard, we propose that adjunctive therapies for protection of normal cell during cancer treatment or reversal of oxidative damage in chemoresistant tumors could be endowed with a promising efficacy by means of exploitation of bothk the properties described above.

Although these results are encouraging, several limitations of this study should be recognized. First, in this study only one cell line (A549) was used; therefore, the results cannot be applied to other lung cancer types or even to other cancer systems. Importantly, the study was performed at a single effective dose (32.6 µg mL−1) of hTRIM21 for gene expression and a full dose-response curve had not been determined for each gene. Further confirmation of Nrf2 pathway activation (eg, by Western blot or immunofluorescence for Nrf2 nuclear translocation) would therefore be useful on the mechanistic level although it is currently inferred from gene expression data. Another point is that Nrf2 plays a double-edged sword role in cancer. Although brief Nrf2 activation can be cytoprotective not only for normal cells but also for early-stage tumors, prolonged or constitutive Nrf2 activation in malignant cells might promote survival and proliferation of cancer cells as well as chemo-resistance. Consequently, the therapeutic window for Nrf2 modulation by agents, such as Theobromine may need to be better defined. Future studies should then investigate whether Theobromine have a protective effect on normal cells from chemotherapy-induced oxidative damage or more likely, it may sensitize cancer cell to chemotherapeutics.

It would be interesting to further explore the upstream signaling events responsible for Nrf2 regulation or possible role of PI3K/Akt or MAPK in Theobromine-mediated effects. Furthermore, assessment of apoptotic markers in the presence and absence of oxidative stress might also help decipher whether Theobromine influence is anti-apoptotic or pro-survival under different conditions (e.g., Bcl-2 families, Caspases... etc.) In summary, this study shows that Theobromine suppresses oxidative stress in A549 lung cancer cells through scavenging of ROS and by upregulating the Nrf2-mediated antioxidant defense pathway. Together with enhanced cell survival and morphology, the results suggest that Theobromine may act as redox-modulating protectants in redox-related pathologies, increasing expression of important antioxidant genes. Being safe and of natural origin, Theobromine can be considered as a promising compound to develop as an adjuvant in therapeutic strategies that aim at counteracting redox imbalance, especially those associated with oxidative stress affected diseases such as lung cancer.

CONCLUSION

We demonstrated that Theobromine at an appropriate concentration (32.6 µg/mL) exerted a protective effect against oxidative

stress in A549 lung cancer cells. Theobromine significantly restored the cell viability, relatively alleviated intracellular ROS production and up-regulated some crucial antioxidant genes expression including Nrf2, HO-1, NQO1, SOD1 and GPx1. It supports the hypothesis that Theobromine mediates its cytoprotective effects through activation of Nrf2 signaling pathway. Theobromine and its chemical versatility as well as capacity for redox modulation make it a very interesting compound, which due to the low cytotoxicity combined with high potential efficacy in antioxidant activity becomes a valid candidate for further research into cancer therapy or prevention strategies.

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