Tempol differently affects cellular redox changes and antioxidant enzymes in various lung-related cells

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Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) is a potential redox agent in cells. The present study investigated changes in cellular reactive oxygen species (ROS) and glutathione (GSH) levels and in antioxidant enzymes, in Tempol-treated Calu-6 and A549 lung cancer cells, normal lung WI-38 VA-13 cells, and primary pulmonary fibroblasts. Results demonstrated that Tempol (0.5–4 mM) either increased or decreased general ROS levels in lung cancer and normal cells at 48 h and specifically increased $O_2^{•−}$ levels in these cells. In addition, Tempol differentially altered the expression and activity of antioxidant enzymes such as superoxide dismutase, catalase, and thioredoxin reductase1 (TrxR1) in A549, Calu-6, and WI-38 VA-13 cells. In particular, Tempol treatment increased TrxR1 protein levels in these cells. Tempol at 1 mM inhibited the growth of lung cancer and normal cells by about 50% at 48 h but also significantly induced cell death, as evidenced by annexin V-positive cells. Furthermore, down-regulation of TrxR1 by siRNA had some effect on ROS levels as well as cell growth inhibition and death in Tempol-treated or -untreated lung cells. In addition, some doses of Tempol significantly increased the numbers of GSH-depleted cells in both cancer cells and normal cells at 48 h. In conclusion, Tempol differentially increased or decreased levels of ROS and various antioxidant enzymes in lung cancer and normal cells, and induced growth inhibition and death in all lung cells along with an increase in $O_2^{•−}$ levels and GSH depletion.

Abbreviations

NSCLC  Non-small cell lung cancer  
SCLC  Small cell lung cancer  
HPF  Human pulmonary fibroblast  
ROS  Reactive oxygen species  
GSH  Glutathione  
Trx  Thioredoxin  
TrxR  Thioredoxin reductase  
SOD  Superoxide dismutase  
NADPH  Nicotinamide adenine dinucleotide phosphate  
H$_2$DCFDA  2',7'-Dichlorodihydrofluorescein diacetate  
DHE  Dihydroethidium  
CMFDA  5-Chloromethylfluorescein diacetate  
MTT  3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide  
FITC  Fluorescein isothiocyanate

The human lung is a structurally multidimensional organ and is susceptible to countless forms of injuries, which are risk factors for developing lung diseases like fibrosis and cancer. As a rule, the physiological restoration process in a healthy lung is constantly active, and usually following injury will repair lung structure and restore function. On the other hand, the progression of lung repair can be pathological, leading to impaired structure and function. Pulmonary fibroblasts (PF) are fundamentally involved in repair and restoration following injuries. During pathological recovery of the lung, sparse or redundant recruitment of fibroblasts can cause...
tissue dysfunction and eventually pulmonary disease. Lung cancer is one of the most common lung diseases and one of the most important contributors to cancer-related mortality worldwide\(^3,4\). Lung cancer consists mainly of either small cell lung cancer (SCLC) or non-SCLC (NSCLC) types, which make up 10% to 13% and 85% to 90% of all lung cancer cases, respectively\(^5,6\). Existing drugs available are still inadequate, and this has prompted a demand for upgraded therapeutic approaches. Among the chemotherapy options tested are cytotoxic drugs that target the cell death signaling process (i.e., apoptosis or necrosis)\(^7,8\).

Reactive oxygen species (ROS) are very unstable oxygen molecules and include hydrogen peroxide (H\(_2\)O\(_2\)), hydroxyl radicals (‘OH), and superoxide anions (O\(_2^-\)) among others. These fundamental molecules are typically considered harmful to cells and tissues. However, ROS are contributory in regulating numerous cellular events such as gene expression, differentiation, and cell proliferation\(^9,10\). ROS, and in particular O\(_2^-\), are constantly generated during mitochondrial oxidative phosphorylation and unequivocally produced by specific oxidases including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase\(^11\). The main degradation pathway to reduce ROS levels employs superoxide dismutases [SODs; intracellular (SOD1), mitochondrial (SOD2), and extracellular (SOD3) isoforms], which metabolize O\(_2^-\) to H\(_2\)O\(_2\). H\(_2\)O\(_2\) is then processed to O\(_2\) and/or H\(_2\)O by catalase or glutathione (GSH) peroxidase\(^12\). GSH is an important antioxidant peptide which can protect cells from toxic insults\(^13\). In addition, thioredoxin (Trx) is a small antioxidant protein (\(\sim 12\) kDa) that has redox-active cysteine residues at its active site\(^14\). The oxidized form of Trx is reduced by NADPH-dependent Trx reductase (TrxR)\(^15\). While Trx1 and TrxR1 are usually localized in the cytoplasm, Trx2 and TrxR2 are found in mitochondria\(^16\). The Trx system is involved in cell survival, tumor development, and inflammatory diseases, particularly lung cancer\(^17-19\).

Oxidative stress due to overproduction of ROS, a lack of antioxidants, or both, can lead to permanent modifications of proteins, lipids, and DNA, leading to cell death and tissue inflammation, consequently resulting in the chronic progression of many diseases including cancer\(^20,21\). More importantly, oxidative stress and chronic inflammation are associated with each other. Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) is a synthetic cyclic nitroxide compound that has been commonly utilized as a contrast material in magnetic resonance spectroscopy\(^22,23\). It undergoes rapid reversible transfer between three forms: nitroxide, hydroxylamine and the oxoammonium cation form\(^24\). Therefore, Tempol is a potential redox agent that may function as a reductive or oxidative mediator depending on its concentration in the cell\(^21\). Treatment with Tempol removes a variety of ROS and diminishes oxidation, subsequently protecting cells and tissues from oxidative injuries\(^25-28\). Our previous reports have shown that Tempol prevents lung cancer cells and juxtaglomerular cells from undergoing cell death in response to cytotoxic insult\(^29,30\). Potential antioxidant mechanisms of Tempol have been proposed to explain these beneficial effects. One possibility is that it may act as a mimic of SOD and reduce the formation of ‘OH either by scavenging O\(_2^-\) or by decreasing the intracellular concentration of Fe (II)\(^30,31,32,33\). However, increasing attention on Tempol in the clinical area has prompted more comprehensive studies on its potential toxicity. Abundant evidence suggests that Tempol interrupts ferritin synthesis and Fe metabolism, which could stimulate cell death\(^30\). The toxicity of Tempol is dose-dependent and preferentially targets cancer cells\(^31\). It has been reported that Tempol at millimolar concentrations inhibits cell growth and induces apoptosis, depending on cell types and origins\(^31,32,33-35\). Moreover, high concentrations of, or extended exposure to Tempol increases ROS levels in juxtaglomerular cells\(^35\), breast cancer cells\(^36\), and ovarian cancer cells\(^33,35\). Thus, in order to clarify the different effects of Tempol as pro-oxidant or antioxidant in cells and tissues, further in depth studies need to be undertaken to elucidate its biological mechanisms.

In this study, alterations to cellular redox molecules such as ROS, GSH, and antioxidant enzymes in response to Tempol were examined in A549 and Calu-6 lung cancer cells, as well as in primary normal human PF (HPF) cells and an SV40-transformed normal HPF line (WI-38 VA-13 subclone 2RA). Moreover, it was examined whether down-regulation of Trx system, especially TrxR1 protein affects cell growth and death as well as ROS levels in Tempol-treated or -untreated lung cancer and normal cells.

**Materials and methods**

**Cell culture.** Human NSCLC A549 adenocarcinoma cells, SCLC Calu-6 cells, and normal lung WI-38 VA-13 subclone 2RA cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Primary normal HPF cells were purchased from PromoCell GmbH (C-12360, Heidelberg, Germany) and used between passages five and ten. Lung cells were kept in a standard humidified incubator at 37 °C with 5% CO\(_2\) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich Co., St. Louis, MO, USA) and 1% penicillin–streptomycin (GIBCO BRL, Grand Island, NY, USA). Cells were grown in 100 mm plastic cell culture dishes (BD Falcon, Franklin Lakes, NJ, USA) and harvested with trypsin–EDTA (Gibco BRL).

**Reagents.** Tempol was obtained from Sigma-Aldrich Co. and dissolved in methanol (Sigma-Aldrich Co.) at 1 M as a stock solution. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was also obtained from Sigma-Aldrich Co. and dissolved in distilled water. SOD and catalase proteins were acquired from Sigma-Aldrich Co. and dissolved in 50 mM potassium phosphate buffer at 4733 U/ml. Activity units of SOD and catalase were defined as the amount of enzyme needed to exhibit 50% dismutation of O\(_2^-\) and to exhibit 50% decomposition of H\(_2\)O\(_2\) to H\(_2\)O and/or O\(_2\) at 25 °C for 10 min, respectively, as previously described\(^36\). TrxR1 protein was also acquired from Sigma-Aldrich Co. All stock solutions were wrapped in foil and kept at 4 °C or -20 °C. Methanol (0.02%) was used as a vehicle control.

**Determination of intracellular ROS and O\(_2^-\) levels.** Overall levels of intracellular ROS including H\(_2\)O\(_2\), ‘OH, and OONO• were measured using a fluorescent probe dye, 2,7’-dichlorodihydrofluorescein diacetate (H\(_2\)DCFDA, Ex/Em = 495 nm/529 nm; Invitrogen Molecular Probes, Eugene, OR, USA), as previously...
Cells were washed twice with cold PBS and then suspended in 200 μL of binding buffer (10 mM HEPES/NaOH (50 mM KH2PO4/50 mM Na2HPO4, pH 7.0) and the reaction was commenced by the addition of 200 mM H2O2 used for the determination of catalase activity. Samples were added to a microcentrifuge tube with assay buffer for 10 min to produce a water-soluble formazan dye. Formazan crystal formation was measured at 450 nm using a microplate reader (BioTekR Instruments Inc. Winooski, VT). SOD activity is expressed in arbitrary units.

Denmark) with enzyme working solution containing a color reagent, and then the plates were incubated at 25 °C for 10 min to produce a water-soluble formazan dye. Formazan crystal formation was measured at 450 nm using a microplate reader (BioTekR Instruments Inc. Winooski, VT). SOD activity is expressed in arbitrary units.

Measurement of cellular catalase activity. The activity of cellular catalase was measured using a catalase assay kit from Sigma-Aldrich Co., as previously described37. In brief, 1 × 10⁶ cells in 60 mm culture dishes (BD Falcon) were incubated with 1 mM Tempol for 48 h. Supernatant samples containing 30 μg protein were used for the measurement of catalase activity. These were added to wells of 96-well microtiter plates (Nunc, Roskilde, Denmark) with enzyme working solution containing a color reagent, and then the plates were incubated at 25 °C for 10 min to produce a water-soluble formazan dye. Formazan crystal formation was measured at 450 nm using a microplate reader (BioTekR Instruments Inc. Winooski, VT). Catalase activity is expressed in arbitrary units.

Measurement of cellular SOD activity. The activity of cellular SOD enzyme was measured using an SOD assay kit (Sigma-Aldrich Co.), as previously described37. In brief, 1 × 10⁶ cells in 60 mm culture dishes (BD Falcon) were incubated with 1 mM Tempol for 48 h. Supernatant samples containing 30 μg protein were used for the determination of catalase activity. These were added to wells of 96-well microtiter plates (Nunc, Roskilde, Denmark) with enzyme working solution containing a color reagent, and then the plates were incubated at 25 °C for 10 min to produce a water-soluble formazan dye. Formazan crystal formation was measured at 450 nm using a microplate reader (BioTekR Instruments Inc. Winooski, VT). SOD activity is expressed in arbitrary units.

Measurement of cellular TrxR1 activity. The activity of TrxR1 was assessed using a thioredoxin reductase assay kit according to the manufacturer's instructions (Sigma-Aldrich Co.). In brief, 1 × 10⁶ cells in 60 mm culture dishes (BD Falcon) were incubated with 1 mM Tempol for 48 h. Supernatant samples containing 30 μg protein were used for the determination of TrxR1 activity. These were added to wells of 96-well microtiter plates (Nunc) with 5,5′-dithiobis-(2-nitrobenzoic acid) from the kit at 25 °C for 1 h. The optical density of each well was measured at 412 nm using a microplate reader (BioTekR Instruments Inc). TrxR1 activity is expressed in arbitrary units.

Transfection of cells with TrxR1 siRNAs. Gene silencing of TrxR1 was performed as previously described39. A control scrambled siRNA duplex [5'-CCU ACCGCCCACAUAUUGCU(dTdT)-3'] and TrxR1 siRNA duplex [5'-GUCCGCUAGGAAGCUU(dTdT)-3'] were purchased from Bioneer Corp. (Daejeon, South Korea). In brief, 2 × 10⁵ cells added to six-well plates (Nunc) were incubated in RPMI-1640 supplemented with 10% FBS. The next day, cells (approximately 20–30% confluence) in each well were transfected with the control, or TrxR1 siRNA duplex [80 pmol in Opti-MEM (GIBCO BRL)] by means of LipofectAMINE 2000, according to the manufacturer's instructions (Invitrogen, Branford, CT). One day later, cells were treated with 1 mM Tempol for 48 h. The transfected cells were used for Western blotting, MTT assays, annexin V-fluorescein isothiocyanate (FITC, Life Technologies, Carlsbad, CA, USA; Ex/Em = 488/519 nm) staining, and ROS measurements.

Cell growth inhibition assay. The effects of Tempol on the growth of lung cells were determined using MTT assays. In brief, 1.5 × 10⁵ cells per well were seeded in 96-well microtiter plates (Nunc) for MTT assays. After incubation with Tempol at 1 mM for 48 h, 20 μL of MTT solution (2 mg/mL in PBS) was added to each well. Plates were then incubated at 37 °C for 3–4 h. Medium in each well was removed by pipetting. Then 100–200 μL of DMSO was added to each well to solubilize formazan crystals. Optical density was measured at 570 nm with a microplate reader (BioTekR Instruments Inc).

Detection of apoptosis. Apoptosis was identified via annexin V-FITC (Life Technologies) as previously described40. Briefly, 3 × 10⁵–5 × 10⁵ cells in six-well plates (Nunc) were incubated with Tempol at 1 mM for 48 h. Cells were washed twice with cold PBS and then suspended in 200 μL of binding buffer (10 mM HEPES/NaOH
pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) at a density of 5 × 10^5 cells/mL at 37 °C for 30 min. After adding Annexin V-FITC (2 μL), cells were analyzed with a FAC Star flow cytometer (BD Sciences).

Detection of intracellular GSH levels. Cellular GSH levels were evaluated using fluorescent probe 5-chloromethylfluorescein diacetate (CMFDA, Ex/Em = 522 nm/595 nm; Invitrogen Molecular Probes), as previously described37. In brief, 1 × 10^6 cells in 60 mm culture dishes (BD Falcon) were treated with Tempol at indicated concentrations (0.5–4 mM) for 48 h. Then, the cells were washed with PBS and incubated with 5 μM CMFDA at 37 °C for 30 min. The mean CMF fluorescence intensity was determined using a FAC Star flow cytometer (BD Sciences). CMF negative (−) staining indicated the depletion of GSH content in cells.

Statistical analysis. The results show the mean of more than two independent experiments (mean ± SD). The data were analyzed using Instat software (GraphPad Prism 5.0, San Diego, CA, USA). A Student’s t-test or one-way analysis of variance with post-hoc analysis using Tukey’s multiple comparison test was applied to determine statistical significance, which was defined as p-values of < 0.05.

Results
Effect of Tempol on intracellular ROS levels in lung cancer and normal cells. To assess the intracellular levels of different kinds of ROS in Tempol-treated cells, H2DCFDA dye was used for general ROS levels and DHE dye was used for O2•− levels. As shown in Fig. 1A, intracellular ROS (DCF) levels were significantly increased in A549 cells treated with 0.5–4 mM Tempol at 48 h. Doses of 1 and 4 mM Tempol increased the levels
by about 170% and 200%, respectively, compared with the control group. In contrast, all tested doses of Tempol significantly decreased ROS (DCF) levels in Calu-6 cells (Fig. 1B). Doses of 1 and 4 mM Tempol decreased the levels by about 65% and 70%, respectively (Fig. 2B). For the normal lung cells, Tempol at 0.5 mM significantly decreased ROS (DCF) levels in WI-38 VA-13 cells at 48 h whereas 2 and 4 mM Tempol significantly increased ROS (DCF) levels in these cells, with 4 mM Tempol increasing the level by about 450% (Fig. 1C). While Tempol at 0.5 and 1 mM significantly decreased ROS (DCF) levels in primary HPF cells at 48 h by about 50%, 2 mM Tempol had no effect (Fig. 1D).

Intracellular $\cdot O_2^-$ (DHE) levels were reduced in A549 cells treated with 0.5 and 1 mM Tempol at 48 h but they were significantly increased at 2 and 4 mM, with 4 mM Tempol increasing by about 500% (Fig. 2A). For the normal lung cells, Tempol at 0.5 mM significantly decreased ROS (DCF) levels in WI-38 VA-13 cells at 48 h whereas 2 and 4 mM Tempol significantly increased ROS (DCF) levels in these cells, with 4 mM Tempol increasing the level by about 450% (Fig. 1C). While Tempol at 0.5 and 1 mM significantly decreased ROS (DCF) levels in primary HPF cells at 48 h by about 50%, 2 mM Tempol had no effect (Fig. 1D).

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**Effects of Tempol on the expression and activity of various antioxidant enzymes in lung cancer and normal cell cells.** Changing ROS levels result from alterations to antioxidant enzyme expression and activity. Thus, it was examined whether Tempol influenced the expression and activity of SOD, catalase, Trx1, and TrxR1 in lung cancer and normal cells at 48 h. As shown in Fig. 3A,B, and Supplementary Fig. 1A, Tempol at 0.5–2 mM did not affect SOD1 protein levels in A549 cells but increased the levels in Calu-6 cells. While 0.5 mM and 1 mM doses of Tempol diminished SOD1 protein levels in WI-38 VA-13 cells, 2 mM slightly increased the protein level (Fig. 3C and Supplementary Fig. 1A). For catalase, apparent differences in expression levels were
Effects of Tempol on the expression levels of SOD1, catalase, Trx1, and TrxR1 in lung cancer and normal cell lines. Exponentially growing cells were treated with indicated concentrations of Tempol for 48 h. Thirty μg of protein extracts from the tested lung cells were resolved by SDS-PAGE gel, transferred to PVDF membranes, and immunoblotted with the designated antibodies. Western blot analysis shows the levels of SOD1, catalase, Trx1, TrxR1, and β-actin in A549 cells (A), Calu-6 cells (B), and WI-38 VA-13 cells (C). The graphs represent the relative expression levels of SOD1, catalase, Trx1 and TrxR1 in A549 cells (A), Calu-6 cells (B), and WI-38 VA-13 cells (C). The expression of β-actin was used as an internal control.

Effects of TrxR1 silencing on cell growth, cell death, and ROS levels in Tempol-treated lung cancer and normal cells. Because treatment with Tempol dose-dependently increased TrxR1 protein levels in A549, Calu-6, and WI-38 VA-13 cells at 48 h (Fig. 3), it was assumed that changes in TrxR1 were closely related to cell growth and death as well as ROS levels. Hence whether gene silencing of TrxR1 using siRNA knock-down alters these cellular effects was examined. TrxR1 siRNA knock-down efficiently decreased protein levels more than 90% in all cell types at 48 h, as compared to scramble siRNA-transfected cells (Fig. 5A and Supplementary Fig. 1C). Transfection of TrxR1 siRNA decreased the growth of A549 cells at 48 h by about 20% in A549, Calu-6, and WI-38 VA-13 cells by about 55%, 50%, and 55%, respectively (Fig. 5B). Tempol at 1 mM also reduced cell growth of TrxR1 siRNA-transfected A549, Calu-6, and WI-38 VA-13 cells by about 50%, 40%, and 50%, respectively (Fig. 5B). Treatment with 1 mM Tempol decreased the growth of scramble siRNA-transfected A549, Calu-6, and WI-38 VA-13 cells by about 50%, 40%, and 50%, respectively (Fig. 5B).

In relation to cell death, transfection with TrxR1 siRNA did not significantly alter the proportion of annexin V-positive cells in untreated A549, Calu-6, and WI-38 VA-13 cells at 48 h (Fig. 6A). However Tempol at 1 mM

![Figure 3](https://doi.org/10.1038/s41598-021-94340-z)
significantly increased the amounts of annexin V-positive cells in both scramble siRNA- and TrxR1 siRNA-transfected A549 cells by approximately 30% and 27%, respectively (Fig. 6A). In addition, Tempol increased the amounts of annexin V-positive cells in scramble siRNA- and TrxR1 siRNA-transfected Calu-6 cells by approximately 24% (Fig. 6A). Interestingly, it augmented the proportion of annexin V-positive cells in scramble siRNA- and TrxR1 siRNA-transfected Wi-38 VA-13 cells by approximately 22% and 17%, respectively, implying that knock-down of TrxR1 attenuates cell death in Tempol-treated Wi-38 VA-13 cells (Fig. 6A).

Effect of Tempol on GSH levels in lung cancer and normal cells. Changes in GSH levels were assessed in lung-related cells using CMF fluorescent dye. As shown in Fig. 7A, Tempol at 0.5 and 1 mM did not augment the number of GSH-depleted cells in Calu-6 cells at 48 h, whereas 2 and 4 mM concentrations significantly increased the number of GSH-depleted cells by about 44% and 74%, respectively. Tempol at 0.5–4 mM significantly increased the number of GSH-depleted cells in A549 cells at 48 h, with 1 and 4 mM Tempol increasing numbers by about 20% and 60%, respectively (Fig. 7B). In WI-38 VA-13 cells, 0.5 and 1 mM Tempol did not affect the number of GSH-depleted cells but 2 and 4 mM concentrations significantly augmented the number of GSH-depleted cells by about 28% and 90%, respectively (Fig. 7C). In addition, Tempol significantly induced GSH depletion in primary HPF cells at 48 h, with 0.5, 1, and 3 mM Tempol increasing the depletion by about 19%, 38%, and 80%, respectively (Fig. 7D).

Figure 4. Effects of Tempol on SOD, catalase, and TrxR1 activity in lung cancer and normal cells. Exponentially growing cells were treated with 1 mM Tempol for 48 h. (A) The graph shows the activity of total SOD in arbitrary units in A549, Calu-6, and WI-38 VA-13 cells. Lysis buffer indicates a negative control in this assay. SOD 10 U indicates a positive control in this assay. (B) The graph shows the activity of catalase in arbitrary units in A549, Calu-6, and WI-38 VA-13 cells. Lysis buffer indicates a negative control in this assay. Catalase 10 U indicates a positive control in this assay. (C) The graph shows the activity of TrxR1 in A549, Calu-6, and WI-38 VA-13 cells. Lysis buffer indicates a negative control in this assay. TrxR1 0.02 μg indicates a positive control in this assay. *p < 0.05 compared with untreated controls.
Discussion

Tempol has been shown to protect cells and tissues from oxidative damage. In contrast, it has also been reported that Tempol enhances inflammation and oxidative damage in numerous cell and tissue models. In addition, millimole concentrations of Tempol or extended exposure to it increases ROS levels in juxtaglomerular cells, breast cancer cells, and ovarian cancer cells. The current study has focused on investigating changes in cellular redox changes and antioxidant enzymes in Tempol-treated lung cancer and normal cells.

Results from the present study showed that ROS (DCF) levels were significantly increased in A549 cells treated with 0.5–4 mM Tempol and in WI-38 VA-13 cells treated with 2 and 4 mM Tempol. However, ROS (DCF) were significantly reduced in Calu-6 cells treated with 0.5–4 mM Tempol and in WI-38 VA-13 cells treated with 0.5 mM Tempol. In addition, Tempol at 0.5 and 1 mM significantly decreased ROS (DCF) in primary HPF cells. These results imply that Tempol can raise or lower ROS (DCF) levels depending on the cell type without significant differences between lung cancer and normal lung cells. Tempol at 1 mM significantly increased the...
activity of catalase in Calu-6 cells, possibly resulting in a decrease in intracellular $\text{H}_2\text{O}_2$ level due to the high conversion from $\text{H}_2\text{O}_2$ to $\text{O}_2$ and $\text{H}_2\text{O}$.

However, the increased activity of catalase in Tempol-treated A549 cells did not reduce ROS (DCF) levels and the decreased activity of catalase in Tempol-treated WI-38 VA-13 cells did not increase ROS (DCF). In addition, Tempol did not apparently alter the expression of catalase in A549 and WI-38 VA-13 cells and decreased catalase in Calu-6 cells. Therefore, the expression levels of catalase in lung cancer and normal cells did not correlate with its enzymatic activity.

Intracellular $\text{O}_2^{•−}$ (DHE) levels were increased in A549, Calu-6, WI-38 VA-13, and primary HPF cells treated with 2 or 4 mM Tempol. Tempol also increased $\text{O}_2^{•−}$ levels in Calu-6 and primary HPF cells at 0.5 and 1 mM but these doses did not augment $\text{O}_2^{•−}$ levels in A549 and WI-38 VA-13 cells. Relatively high concentrations of Tempol appeared to increase $\text{O}_2^{•−}$ in each of the cell lines. Tempol is a mimic of SOD which metabolizes $\text{O}_2^{•−}$ to $\text{H}_2\text{O}_2$. Similarly, 1 mM Tempol up-regulated the activity of SOD in Calu-6 cells at 48 h. However, Tempol did not affect the activity of SOD in WI-38 VA-13 cells. Tempol at 1 mM even reduced the activity of SOD in A549 cells. Treatment with Tempol increased SOD1 protein levels in Calu-6 cells but not in A549 cells. Contrarily, at 1 mM it decreased the levels in WI-38 VA-13 cells. Therefore, the expression levels of SOD1 in A549 and WI-38 VA-13 cells were not related to its enzymatic activity. It was speculated that the increased amount of intracellular $\text{O}_2^{•−}$ in cells in response to Tempol may have been originated from mitochondria. Numerous reports have demonstrated that Tempol mediates its toxicity via the disruption of mitochondrial function, suggesting that Tempol's effects on mitochondria may result in excessive production of $\text{O}_2^{•−}$, causing oxidative stress and consequently apoptosis. In fact, 2 and 4 mM of Tempol strongly increased mitochondrial $\text{O}_2^{•−}$ levels in WI-38 VA-13 cells at 48 h, by approximately sixfold and 22-fold, respectively (Supplementary Fig. 3).

Figure 7. Effects of Tempol on intracellular GSH depletion in lung cancer and normal cells. Cells in the exponential growth phase were incubated with the indicated concentrations of Tempol for 48 h. Intracellular CMF (GSH) levels in various lung cells were measured using a FAC Star flow cytometer. The graphs indicate the percentages of (-) CMF (GSH-depleted) in A549 cells (A), Calu-6 cells (B), WI-38 VA-13 cells (C), and primary HPF cells (D). *p < 0.05 compared with untreated controls.
cancer and normal cells, death from Tempol was more correlated with changes in O$_{2}^{-}$ levels than ROS (DCF) levels. The apparent diverse effects of Tempol in different conditions and in different cells could be explained by assuming that each cell has a different basal activity or metabolism of mitochondria and a variety of antioxidant enzymes present in the cytoplasm or other organelles.

The Trx system consists of Trx, TrxR, and NADPH and is a fundamental enzymatic complex that preserves cellular redox homeostasis. Trx1 and/or TrxR1 are overexpressed in various pulmonary diseases including cancer. Hence, it is plausible that the Trx system is a promising target for treatment of lung cancer. Tempol significantly decreased the activity of TrxR1 in A549, Calu-6, and WI-38 VA-13 cells. However, TrxR1 protein levels were increased in all these lung cells. Thus, the increased activity of TrxR1 in Tempol-treated lung cells is more likely to be attributed to other factors such as Trx and Trx-interacting proteins rather than simple expression levels. In addition, Tempol treatment increased Trx1 protein expression in A549 cells and WI-38 VA-13 cells but not in Calu-6 cells. Further research is needed on the mechanism underlying alterations in the Tempol-mediated Trx system.

Treatment of Tempol at 1–5 mM inhibits cell growth and induces apoptosis in various cancer cells. Likewise, 1 mM Tempol reduced the growth of scramble siRNA-transfected A549 and Calu-6 lung cancer cells, and WI-38 VA-13 normal cells by about 55%, 30%, and 55% at 48 h, respectively. Moreover, Tempol at 1 mM also significantly increased the amounts of annexin V-positive cells in scramble siRNA-transfected A549, Calu-6, and WI-38 VA-13 cells by about 30%, 24%, and 22% at 48 h, respectively. Therefore, Tempol is likely to induce lung cell death via apoptosis and/or necrosis. There was no significant difference in the susceptibility to Tempol among these lung cancer and normal cells. TrxR1 silencing itself did not influence the growth of Calu-6 and WI-38 VA-13 cells compared with scramble siRNA-transfected control cells but it did inhibit the growth of A549 control cells by about 20%. Transfection of TrxR1 siRNA did not significantly alter the growth inhibition in both Tempol-treated lung cancer and normal cells. Thus, reduced TrxR1 protein level was involved in growth inhibition only in A549 cells among these lung cells and did not affect growth inhibition in these lung cells treated with Tempol. In addition, TrxR1 siRNA alone did not increase the amounts of annexin V-positive cells in A549, Calu-6, and WI-38 VA-13 control cells. Furthermore, knock-down of TrxR1 slightly decreased cell death in Tempol-treated A549 cells and significantly attenuated cell death in Tempol-treated WI-38 VA-13 cells. These results suggest that TrxR1 silencing does not have a direct effect on cell survival in Tempol-untreated control lung cells, but has a differential effect on cell survival in various lung cells treated with Tempol.

A549 cells transfected with TrxR1 siRNA showed slightly decreased ROS (DCF) levels at 48 h while transfected Calu-6 cells showed slight increases. TrxR1 siRNA reduced the increased levels of ROS (DCF) in Tempol-treated A549 cells whereas silencing partially recovered the decreased levels of ROS (DCF) in Tempol-treated Calu-6 cells. In WI-38 VA-13 cells, TrxR1 siRNA did not alter ROS (DCF) levels regardless of 1 mM Tempol treatment. It has been reported that Trx or TrxR1 could regulate the basal cellular metabolism of glycolysis, tricarboxylic acid cycle, and mitochondria to produce adenosine triphosphate (ATP) or NAD(P)H. In addition, there are potentially harmful interactions between Tempol and enzyme complexes, which are involved in glucose transport, glutamine metabolism and mitochondrial ATP production. Therefore, further studies are needed on the difference in cellular ROS levels in lung cancer and normal cells following downregulation of TrxR1 and treatment with Tempol.

Depletion of GSH promotes cell death and this has been reported to occur in Tempol-treated leukemia and cervical cancer cells. Similarly, the present study demonstrated an increased proportion of GSH-depleted cells in A549, Calu-6, WI-38 VA-13, and primary HPF cells treated with 2 or 4 mM Tempol. In addition, lower doses of Tempol (0.5 and 1 mM) significantly induced GSH-depleted cells in Calu-6 and primary HPF cells. These results support the notion that the induction of cell death was inversely proportional to GSH content. However, 1 mM Tempol, which showed cell growth inhibition and cell death in A549 and WI-38 VA-13 cells, did not significantly augment the number of GSH-depleted cells in these cells. As there was little difference in susceptibility to cell death to Tempol between lung cancer and normal cells, the effect of GSH depletion by Tempol was not significantly different between lung cancer and normal cells. According to the present study, GSH content is a crucial factor or indicator of cell death induced by Tempol, but its content alone is not sufficient to accurately predict cell death.

In summary, the present results have shown that Tempol at 0.5–4 mM could either increase or decrease ROS (DCF) levels in lung cancer and normal cells and this drug specifically increased O$_{2}^{-}$ levels and GSH depletion. Tempol at 1 mM inhibited the growth of lung cancer and normal cells and also induced their cell death. There was no significant difference in Tempol sensitivity between lung cancer and normal cells. In addition, Tempol had other effects on the expression and activity of antioxidant enzymes, especially TrxR1 in lung cancer and normal cells. Furthermore, down-regulation of TrxR1 partially affected cell growth and death, as well as ROS (DCF) levels in both Tempol-untreated or -treated cells, depending on the cell type. The current results provide valuable information for understanding the cellular effects of Tempol on lung cancer and normal cells with regard to cell growth inhibition and cell death, as well as redox states (ROS and GSH levels) and various antioxidant pathways.

**Ethics declarations.** The material in this paper has not been published or is not under active consideration by another journal. The research was conducted in accordance with the declaration of Helsinki.

**Data availability**

Data collected during the present study are available from the corresponding author upon reasonable request.
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**Author contributions**

W.-H.P. is the only author who planned and conducted all experiments and wrote the present paper.

**Competing interests**

The author declares no competing interests.

**Additional information**

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