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Differential concentration-specific effects of caffeine on cell viability, oxidative stress, and cell cycle in pulmonary oxygen toxicity *in vitro*

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ABSTRACT

Caffeine is used to prevent bronchopulmonary dysplasia (BPD) in premature neonates. Hyperoxia contributes to the development of BPD, inhibits cell proliferation and decreases cell survival. The mechanisms responsible for the protective effect of caffeine in pulmonary oxygen toxicity remain largely unknown. A549 and MLE 12 pulmonary epithelial cells were exposed to hyperoxia or maintained in room air, in the presence of different concentrations (0, 0.05, 0.1 and 1 mM) of caffeine. Caffeine had a differential concentration-specific effect on cell cycle progression, oxidative stress and viability, with 1 mM concentration being deleterious and 0.05 mM being protective. Reactive oxygen species (ROS) generation during hyperoxia was modulated by caffeine in a similar concentration-specific manner. Caffeine at 1 mM, but not at the 0.05 mM concentration decreased the G2 arrest in these cells. Taken together this study shows the novel funding that caffeine has a concentration-specific effect on cell cycle regulation, ROS generation, and cell survival in hyperoxic conditions.

1. Introduction

Supplemental oxygen is often used in the treatment of lung diseases such as respiratory distress syndrome (RDS) in premature neonates. Exposure to high concentrations of inhaled oxygen (hyperoxia) combined with other factors such as mechanical ventilation, sepsis, etc. leads to lung injury and development of bronchopulmonary dysplasia (BPD) in these fragile patients [1].

Exposure to hyperoxia leads to increased production of reactive oxygen species (ROS), inhibition of cell proliferation, cell cycle arrest and eventually cell death [2]. Hyperoxia leads to activation of different cell cycle checkpoints depending on factors such as the cell type and the p53 status of the cell. Cells with wild type p53 arrest in the G1 phase and cells with deficient p53 arrest in the S or G2 phase [3–6]. Repair of genotoxic effects of hyperoxia is essential for subsequent tissue recovery.

Caffeine has been observed to decrease the incidence of BPD in premature neonates [7]. The mechanisms responsible for the protective effect of caffeine in pulmonary oxygen toxicity remain unknown. Variable concentrations ranging from micromolar to high millimolar have been used in studies evaluating effects of caffeine on cell cycle progression *in vitro*. The goal of this study was to determine the effects of caffeine, at concentrations that are clinically relevant in BPD patients on pulmonary epithelial cells exposed to hyperoxia, *in vitro*. We tested the hypothesis that caffeine will elicit concentration-specific effects on cell cycle progression, oxidative stress, and viability in human and murine pulmonary epithelial (A549: intact p53 and MLE 12: disrupted p53) cell lines exposed to hyperoxia. We used 0.05 mM (equivalent to 10 mg/kg, molecular weight of caffeine: 194.19 g/mol) and 0.1 mM (equivalent to 20 mg/kg) concentrations to model the dose ranges used clinically in premature neonates. In the current study, we demonstrate that caffeine has differential effects on cell cycle progression, cell viability, and oxidative stress in pulmonary epithelial cell lines exposed to hyperoxia depending on the concentration.

2. Materials and methods

2.1. Cell culture and caffeine preparation

A549 human lung epithelial cells and MLE 12 SV40 transformed mouse epithelial cells were obtained from the American Type Culture Collection (Rockville, MD). Both of these cell lines have type II alveolar epithelial cell characteristics. A549 cells have an intact p53-dependent G1 checkpoint. MLE 12 cells express the SV40 large

Abbreviations: BPD, bronchopulmonary dysplasia; RDS, respiratory distress syndrome; ROS, reactive oxygen species.

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T antigen, which binds to p53 leading to uncontrolled cellular proliferation and disrupts the p53 mediated G1 checkpoint [30]. Cells were cultured in DMEM/F-12, 50/50, (Cell Gro, Manassas, VA) supplemented with 10% fetal bovine serum, 50 U penicillin/ml, and 50 μg/ml streptomycin in a 5% CO_2/95% air atmosphere at 37 °C. Caffeine was purchased from Sigma Aldrich (St. Louis, MO, USA) and varying concentrations of caffeine (0.05, 0.1 and 1 mM) were prepared in 1× Dulbecco's Phosphate-Buffered Saline (Cell Gro, Manassas, VA, USA). We used 0.05 mM (≅10 mg/kg) and 0.1 mM (≅20 mg/kg) concentration to model the dose ranges used clinically in premature neonates. All cells were routinely passaged every 3 days.

2.2. Exposure of cells to hyperoxia

Hyperoxia experiments were conducted in a Plexiglas sealed chamber into which a mixture of 95% O_2 and 5% CO_2 was circulated continuously. The chamber was placed in a Forma Scientific water-jacketed incubator at 37 °C. Once the O_2 level inside the chamber reached 95%, the cells were placed inside the chamber for the desired length of time (up to 72 h). For the study of caffeine effects, exponentially growing cells were cultured for 24 h in medium. Cells were exposed to caffeine for 4 h at 5% CO_2/95% air atmosphere before subjecting them to hyperoxia or control conditions. For each protocol described below, three or four independent experiments were performed.

2.3. Trypan blue exclusion for cell viability

Cells were treated with caffeine as described before and were exposed to room air or hyperoxia for up to 72 h. After harvesting, they were diluted 1:1 in 0.4% Trypan Blue dye (Cat # 145–0013) from Bio-Rad laboratories Inc. 10 μl was loaded on counting slides (Cat # 145–0011). TC20™ Automated Cell Counter (Bio-Rad laboratories Inc.) was used to obtain the number of total cells and live cells.

2.4. Measurement of ROS generation

The ROS-Glo™ Assay (Promega Inc. Madison, WI) was used to measure the level of hydrogen peroxide (H_2O_2), directly in cell culture according to the manufacturer's recommendations. Cells were plated at a density of 100,000 cells/well in a 96 well plate and incubated overnight for attachment. Cells were treated with varying caffeine concentrations at 0.05 mM, 0.1 mM and 1 mM. Plates were placed in hyperoxia or normoxia for 6 h, 12 h and 24 h. H_2O_2 substrate solution was added to the plates 6 h before read and the plates were replaced in hyperoxia chamber. The detection solution was added to the plates 20 min before each read and incubated at room temperature and relative luminescence was recorded using SpectraMax M3 microplate reader (Molecular Devices LLC, Sunnyvale, CA).

2.5. Cell cycle analysis

Asynchronously proliferating cultures at room air or after hyperoxia exposure with or without caffeine were subjected to flow cytometry analysis for assessment of the cell cycle. Quantitative DNA content analysis in cells was performed using the nucleic acid stain propidium iodide followed by flow cytometry (Abcam, Cat. # ab139418). Briefly, cells were grown on six-well plates to 60–70% confluence, after which they were treated with caffeine and exposed to room air or hyperoxia for up to 72 h. Cells were harvested in single cell suspension and fixed in 66% ethanol at 4 °C. Cells were stained with propidium iodide and RNase A and incubated at 37 °C for 30 min. Cell-cycle distribution was determined by using flow cytometry (FACSort, Becton Dickinson) and ModFit LT software (Verity Software House, Topsham, ME) giving us the percentage of cells in different cell cycle stages.

2.6. In-Cell ELISA measuring Cdk2 (pTyr15) and Histone H3 (pSer10)

We used quantitative immunocytochemistry (In-Cell ELISA Assay Kit purchased from Abcam (Cat # ab140363) to measure levels of Cdk2 protein phosphorylated Tyr15 (elevated in the G1/S phase) and Histone H3 protein phosphorylated Ser10 (elevated in G2/M phase) levels in A549 and MLE 12 cells exposed to room air or hyperoxia for 24, 48 or 72 h. Phosphorylation of Cdk2 at Tyr15 indicates that a cell is at the G1/S transition [31]. Phosphorylation of Histone H3 at Ser10 is tightly correlated with chromosome condensation during mitosis [32]. Hence, Histone H3 pSer10 signal indicates a mitotic cell with condensed DNA.

2.7. Statistical analysis

Results are reported as means ± standard error of the mean (SEM). Data were analyzed using 2-way analysis of variance (the main effects were: caffeine concentration and hyperoxia), followed by Bonferroni posttests for comparisons against control conditions using GraphPad version 5. Significance was assigned for $P < 0.05$.

3. Results

3.1. Effects of caffeine on cell viability following hyperoxia exposure in A549 or MLE 12 cells

Cell viability was measured at room air and following 24–72 h of hyperoxia exposure in the presence (0.05, 0.1 and 1 mM) or absence of caffeine. A549 cells were relatively resistant to hyperoxia with no significant change in viability up to 72 h. Cells (Fig. 1A) treated with 1 mM caffeine showed a significant decrease in viability at 72 h compared to no treatment. Treatment with caffeine at 0.05 and 0.1 mM concentration showed no difference in cell viability compared to no caffeine treatment. MLE 12 cells (Fig. 1B) were more sensitive to hyperoxia exposure than A549 cells. There was progressive decrease in viability with a significant decrease at 72 h compared to room air. Treatment with 1 mM caffeine accelerated this process with cells showing decreased viability at the 24 and 48 h time point. Compared to no treatment, caffeine at 1 mM concentration significantly decreased in viability in MLE 12 cells exposed to hyperoxia at the 24 and 72 h time point.

3.2. Effects of caffeine on ROS levels in A549 or MLE 12 cells exposed to hyperoxia

In A549 cells (Fig. 2A), there was a significant increase in H_2O_2 levels at 6 and 12 h after hyperoxia exposure. Treatment with caffeine at 0.05 mM concentration decreased (6 and 12 h) and 1 mM concentration increased (24 h) H_2O_2 levels compared to cells with no caffeine treatment. With 0.1 and 1 mM caffeine treatment, the H_2O_2 levels were elevated at the 24 h time point compared to room air. MLE 12 cells (Fig. 2B) showed a similar elevation in H_2O_2 levels compared to room air at 6 h. Attenuation of H_2O_2 levels was seen at both 6 and 12 h with caffeine treatment at a concentration of 0.05 mM when compared to cells with no caffeine treatment.

3.3. Effects of caffeine on cell cycle progression in A549 or MLE 12 cells exposed to hyperoxia

Exposure of A549 cells to hyperoxia (Fig. 3A, B and C) activated the G1 checkpoint, with more cells retained in G1 at 72 h and a sig-

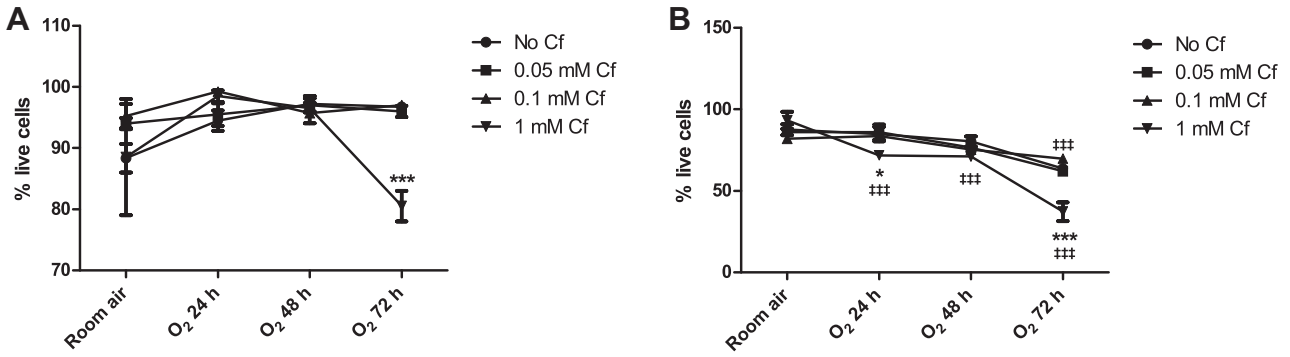

Fig. 1. Effects of hyperoxia and caffeine (Cf) on cell viability. A549 (A) and MLE 12 (B) cells exposed to room air (room air – 5% CO_2) and 24, 48, or 72 h of hyperoxia (95% O_2–5% CO_2) in the presence (0.05 mM or 1 mM) or absence of caffeine were subjected to trypan blue exclusion as described in materials and methods. Values are means ± SEM of 3 independent experiments. Significant differences between No Cf and Cf groups are indicated by $^{*}P < 0.05$ and $^{***}P < 0.001$. Significant differences between corresponding room air values are indicated by $^{‡‡‡}P < 0.001$.

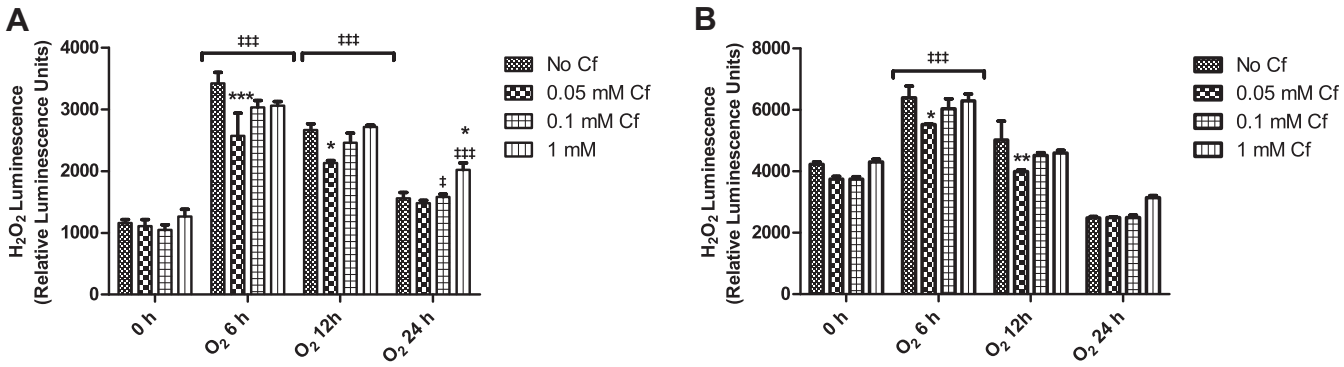

Fig. 2. Effects of hyperoxia and caffeine (Cf) on reactive oxygen species (H_2O_2) production. A549 (A) and MLE 12 (B) cells exposed to room air (room air – 5% CO_2) and 24, 48, or 72 h of hyperoxia (95% O_2–5% CO_2) with No Cf, 0.05 mM Cf or 1 mM Cf were subjected to the ROS-Glo™ luminescent H_2O_2 assay. Values are means ± SEM of 3 independent experiments. Significant differences between No Cf and Cf groups are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Significant differences between corresponding room air values are indicated by $^{‡}P < 0.05$ and $^{‡‡‡}P < 0.001$.

nificant decrease in the number of cells in S and G2 phase. At 48 h, there was decrease in percentage of cells in G1 and a corresponding increase in cells in S and G2 phase of the cell cycle. At 0.05 mM, caffeine decreased the G1 retention at 72 h and had more cells in the S phase compared to no caffeine. On the other hand, at 1 mM concentration, caffeine increased the fraction of cells in G1 (48 h), and decreased the accumulation of cells in G2 (48 and 72 h) compared to other groups (0.05 mM and no caffeine).

In MLE12 cells, hyperoxia significantly decreased the percentage of cells in G1 and increased the percentage of cells in S (24 h) and G2 phase (48 and 72 h) (Fig. 3D, E and F). At 0.05 mM concentration, the effect on cell cycle progression was similar to cells with no caffeine. Caffeine at 1 mM concentration markedly reduced the number of cells in G2 phase at 24, 48 and 72 h time point. This was accompanied with an increase in the number of cells in G1 phase (24, 48 and 72 h) and S phase at 72 h.

3.4. Effects of caffeine on Cdk2 (pTyr15) and Histone H3 (pSer10) in A549 and MLE 12 cells exposed to hyperoxia

In A549 cells (Fig. 4A), there was a decrease in Cdk2 (pTyr15) levels at 24, 48 and 72 h time point in cells with no caffeine, 0.05 and 0.1 mM caffeine compared to room air levels. With 1 mM caffeine this decrease was not seen. Histone H3 (pSer10) levels (Fig. 4B) showed no change in cells with no caffeine, 0.05 and 0.1 mM caffeine, but with 1 mM concentration, there was a significant increase at 72 h time point compared both to room air levels and other cell populations.

In MLE 12 cells, there was a significant increase in Cdk2 (pTyr15) expression (Fig. 4C) at 72 h time point in all cell subpopulations, with the 1 mM concentration showing an earlier increase at the 24 and 48 h time points compared to room air levels. Cells treated with 1 mM caffeine had higher (24 and 48 h) and those treated with 0.05 and 0.1 mM caffeine had lower expression (72 h) of Cdk2 (pTyr15) compared to cells with no caffeine. There was a decrease in Histone H3 (pSer10) levels (Fig. 4D) with hyperoxia exposure except in cells treated with 1 mM caffeine, which showed a significant increase in levels at 72 h compared to room air and no caffeine group. Caffeine did not alter viability, ROS levels, or cell cycle profiles of cultures exposed to room air.

4. Discussion

Hyperoxia leads to lung injury in animal models and causes growth arrest and eventually cell death in cultured cells [2,8,9]. It contributes to the development of diseases such as BPD in premature neonates. Caffeine has been used in neonates to decrease the incidence BPD [7]. Despite being one of the most commonly used drugs in neonates, the mechanisms responsible for the protective effect of caffeine in premature infants especially in the setting of hyperoxic lung injury are not known. A recent study in neonatal mice has shown deleterious effects of caffeine on alveolar development in a murine model of hyperoxia-induced alveolar hypoplasia [10]. In this study, we provide evidence that caffeine has a differential concentration-specific effect on cell cycle progression, checkpoint activation, cell viability and oxidative stress

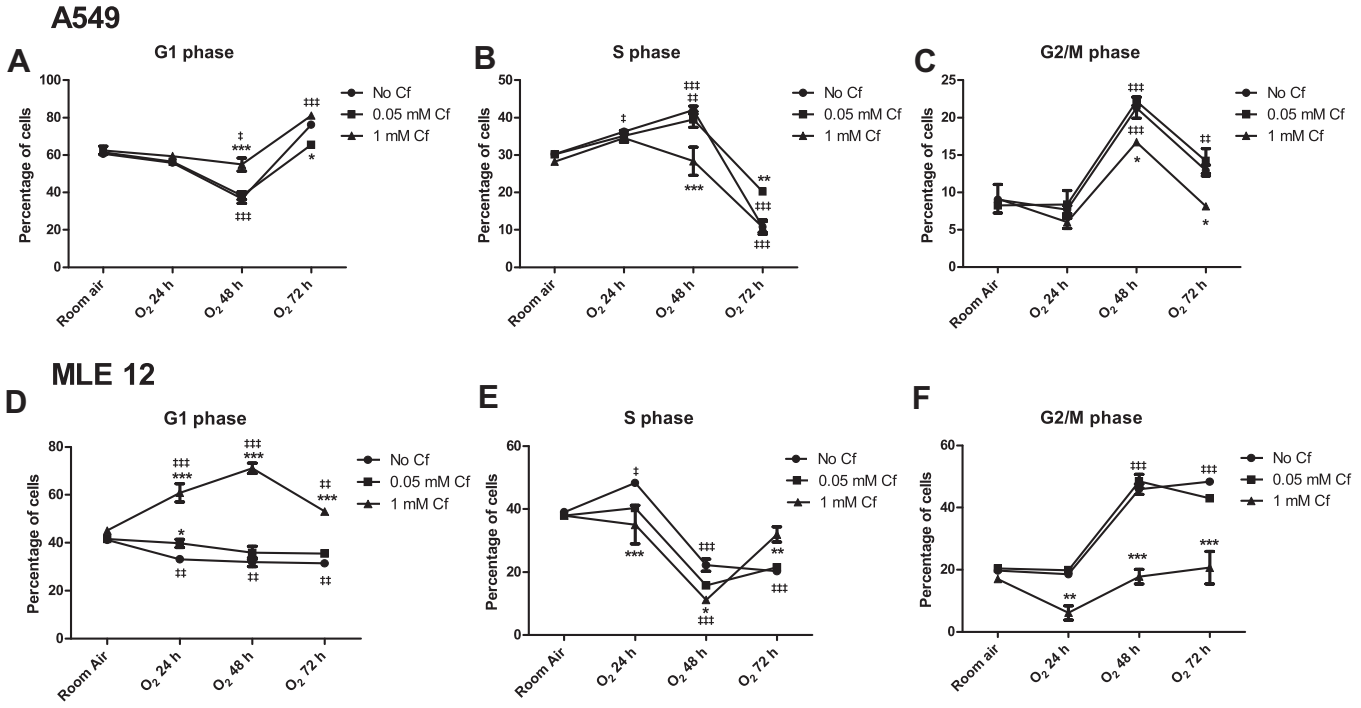

Fig. 3. Effect of hyperoxia and caffeine on A549 and MLE12 cell cycle distribution. A549 (A, B and C) and MLE 12 (D, E and F) cells were cultured in room air or hyperoxia in the presence (0.05 mM or 1 mM) or absence of caffeine (Cf). The percentages of cells in G1, S, and G2 were quantified using flow cytometry. Values are means ± SEM of 3 independent experiments. Significant differences between no caffeine and caffeine groups are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Significant differences between corresponding room air values are indicated by $^{‡}P < 0.05$, $^{‡‡}P < 0.01$ and $^{‡‡‡}P < 0.001$.

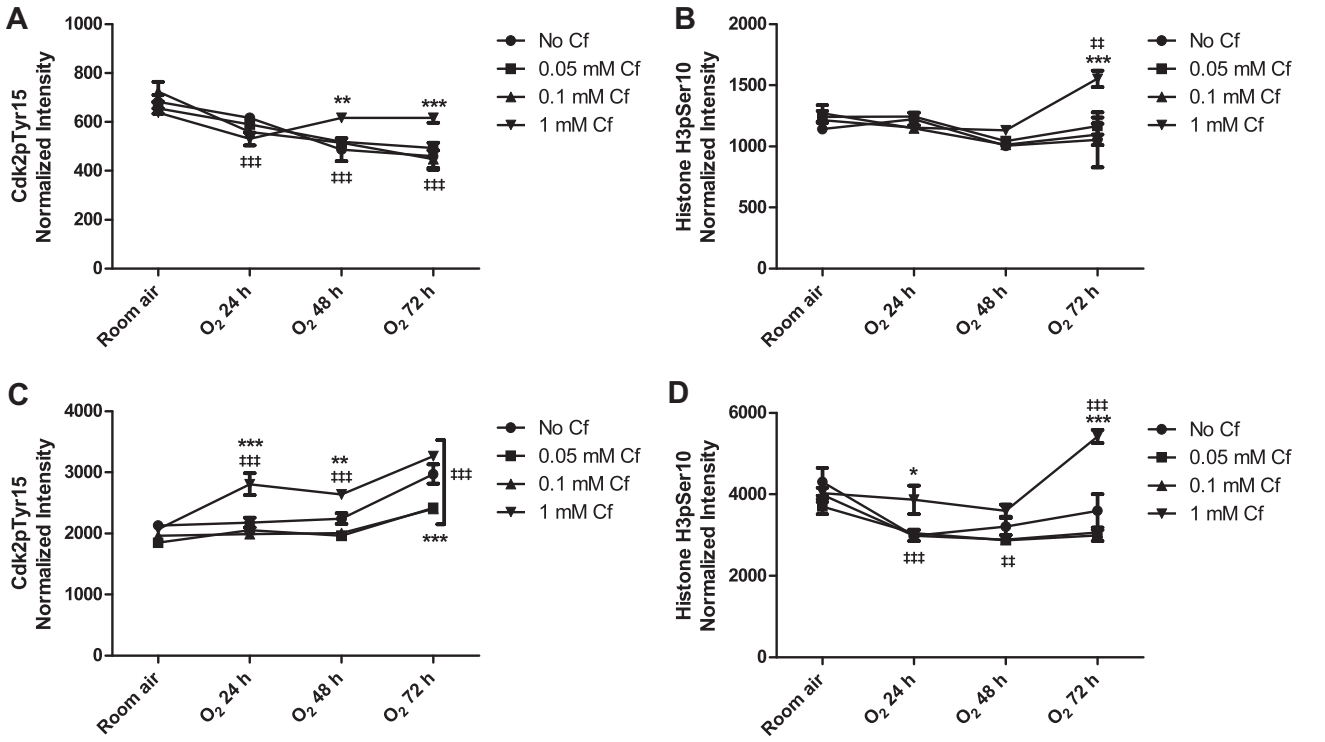

Fig. 4. Quantitative immunocytochemistry to study effects of hyperoxia and caffeine (Cf) on cell cycle. A549 (A and B) and MLE 12 (B and C) cells exposed to room air (room air – 5% CO_2) and 24, 48, or 72 h of hyperoxia (95% O_2–5% CO_2) with no caffeine 0.05 mM or 1 mM caffeine. Levels of Cdk2 (pTyr15) and Histone H3 (pSer10) were measured in A549 (A and B) and MLE 12 (C and D) cells using the In cell ELISA technique as described in materials and methods. Values are means ± SEM of 3 independent experiments. Significant differences between no caffeine and caffeine groups are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Significant differences between corresponding room air values are indicated by $^{‡‡}P < 0.01$ and $^{‡‡‡}P < 0.001$.

in human and murine pulmonary epithelial cell lines with intact (A549) and perturbed (MLE12) p53 status under hyperoxic conditions. Our findings suggest that at doses clinically used in premature neonates (20 mg/kg loading and 5–10 mg/kg/day maintenance), caffeine may ameliorate lung injury by decreasing ROS production. To our knowledge, this is the first study showing that caffeine has a differential concentration-specific effect on modulation of cell cycle checkpoints and ROS generation in pulmonary oxygen toxicity in human and murine pulmonary epithelial cells *in vitro*.

Hyperoxia causes DNA damage and is genotoxic [2,11,12]. These changes cause the cell to arrest at different checkpoints to allow for DNA repair to occur before continuing proliferation. Cells with intact p53 have been shown to arrest in the G1/S phase when exposed to hyperoxia. Increase in p53 after hyperoxia exposure has been documented both *in vivo* and *in vitro* models [13,14]. p53 induces p21 which in turn inhibits cyclin dependent kinases including cdk2 [15,16] preventing the proliferation of oxygen exposed cells thus possibly contributing to alveolar hypoplasia, which is the hallmark of BPD [17]. Cells with perturbed/mutated p53 arrest in the G2/M phase and are also more susceptible to oxygen toxicity [3,18]. In this study, A549 cells exhibited arrest in the G1 phase and the MLE12 cells arrested in the G2 phase. MLE12 cells showed significantly decreased cell viability compared to A549 cells following hyperoxia exposure.

The protective effect of caffeine against oxidant-induced damage has been documented both *in vivo* and *in vitro*. Caffeine (single dose 10 mg/kg) decreased oxidative-stress derived DNA damage in the dentate gyrus in newborn rat pups exposed to hyperoxia [19]. The antioxidant effect of lower concentrations (0.01 and 0.1 mM) of caffeine has been reported in human skin fibroblasts and alveolar macrophages [20,21]. Caffeine was shown to be an efficient hydroxyl radical scavenger *in vitro* [22]. In our study, caffeine had different concentration-specific effects on cellular oxidative stress in alveolar epithelial cells exposed to hyperoxia with the lower concentration decreasing and higher concentration increasing H_2O_2 levels in both MLE 12 and A549 cells. This could explain the better pulmonary outcomes in babies treated with caffeine in the neonatal period when they are exposed to increased oxidative stress.

The effects of caffeine on the G2 phase have been widely documented [23]. Most research indicates that caffeine abrogates the DNA damage-induced G2 arrest, decreasing the time for DNA repair, and the continued progression of these damaged cells through mitosis leads them to apoptosis [2,23,24]. Caffeine increases the potency of DNA damaging agents, especially in p53 deficient cells [25,26]. In an *in vitro* model of oxygen toxicity with A549 and MLE 15 cell lines, caffeine (2 mM concentration) eliminated the G2 arrest and increased cell death [3]. The possible mechanisms involved could be activation of Cdc2 (cdk1) and Cdc25C [23]. We show that at high (1 mM) concentration, caffeine abrogated the hyperoxia-induced G2 arrest in both A549 and MLE12 cells and decreased cell survival and this effect was more pronounced in MLE 12 cells with a perturbed p53 pathway, however this effect was not seen at 0.05 mM concentration.

The decrease in Cdk2 (pTyr15) levels in A549 cells exposed to hyperoxia with no or 0.05 mM caffeine, compared to room air, can be explained by the up regulation and subsequent binding of p21 with cdk2 [16,27]. This effect was not seen with 1 mM caffeine treatment. The effect in MLE 12 cells was different. Cdk2 (pTyr15) was increased at 72 h compared to room air levels. The increase in Cdk2 (pTyr15) levels in cells with mutated p53 with hyperoxia was also observed in asynchronous human T47D-H3 cells [4].

There was a significant increase in levels of Histone H3 (pSer10) at 72 h time point in both A549 and MLE 12 cells with 1 mM caffeine treatment, which indicates a mitotic cell with condensed DNA. Both the no caffeine and 0.05 mM caffeine treated MLE 12 cells showed decreased levels of Histone H3 (pSer10) compared to room air consistent with G2/M phase arrest. Gabrielli et al. (2007) showed that p21-activated kinase 1, which has an anti-apoptotic function in spindle checkpoint-arrested cells, is a target for caffeine inhibition and that caffeine at high concentration induced apoptosis in cells arrested in this phase through this mechanism [28].

We used 95% oxygen for hyperoxia exposure in this study. The effect of clinically relevant oxygen concentrations (40–60%) on cell cycle and its modulation by drugs such as caffeine needs further investigation. The changes in cell cycle progression could be different at different levels of hyperoxia exposure [29].

In summary, we demonstrate that caffeine has a differential concentration-specific effect on oxidative stress, cell viability, and cell cycle progression in these cells. Caffeine at high concentrations abrogates the G2 arrest and increases cell death, whereas at lower and clinically relevant concentration it has no effect on the G2 arrest and has antioxidant properties. The present study provides an insight into the possible mechanism of protective effect of caffeine in diseases such as BPD, where hyperoxia exposure contributes to their pathogenesis.

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