



## Original article

## Toxicological interaction between tobacco smoke toxicants cadmium and nicotine: An in-vitro investigation

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## ABSTRACT

Cigarettes and other tobacco products are used to obtain nicotine that is responsible for their stimulating effects. However, a lot of other organic and inorganic chemicals are also released along with nicotine. Cadmium (Cd) is one of the several heavy metals that are health hazards and is one of the inorganic elements released in tobacco smoke. The *in-vitro* investigation focused on exploring the effects of nicotine hydrogen tartrate (NHT) and cadmium (Cd) and their toxic interactions in the A549 cell line. In cell viability assay NHT exhibited its IC<sub>50</sub> at 11.71 mM concentration, and the IC<sub>50</sub> of Cd was found to be 83 μM after a 24 h exposure. Toxic effects of NHT (5 mM and 10 mM), Cd (50 μM and 100 μM), and their combination were also investigated by flowcytometry. The investigation included apoptotic and necrotic events, the effect on different cell cycle phases, and generation of reactive oxygen species by NHT, Cd, and their combination of different concentrations. Data reveal evident toxic effects of NHT, Cd, and NHT + Cd. It also indicates that the toxic interaction of NHT and Cd is not additive and appears to be minimal when compared with NHT or Cd exposures alone.

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## 1. Introduction

The use of tobacco products not only causes health problems but also raises environmental concerns. In both of the cases, the problems can be attributed to thousands of chemicals present in tar, a sticky, brownish substance released from the burning tobacco, in addition to other gaseous and volatile chemicals. Most of the visible part or particulate phase of the tobacco smoke is constituted by tar (Smith and Fischer, 2001). However, tobacco is used mainly for nicotine owing to its stimulant effects, however, the exposure is not stand-alone. It is accompanied by thousands of chemicals released as byproducts during tobacco burning. Most of these chemicals are present in tar, others include other gaseous

and volatile chemicals. This may not be the case with chewable tobacco, but the hazard remains similar to that of smoking tobacco. The tar contains a large number of organic and inorganic chemicals, including dozens of carcinogens and toxic heavy metals (Pfeifer et al., 2002). Tobacco smoke is one of the major sources of exposure to cadmium (Cd), a toxic heavy metal that is listed by WHO along with other chemicals of public health concern.

Biological effects of nicotine are not only limited to stimulation of CNS but also include several other toxic manifestations including cardiovascular effects (Benowitz and Burbank, 2016). Researchers also speculated that nicotine may have a role in carcinogenesis and developmental and reproductive toxicity (Greene and Pisano, 2019; Price and Martinez, 2019). However, most of the toxic effects, in the case of tobacco smoking, are attributed to the combination of chemicals present in the particulate matter of the smoke. Electronic cigarettes release a less complex mixture of chemicals owing to controlled formulation and manufacturing of nicotine cartridges. Initially, they were presumed as a safer alternative to traditional tobacco smoking due to the presence of very few chemicals, including nicotine, and the absence of the more notorious tar, which may reduce the exposure to noxious agents

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(Goniewicz et al., 2017; McAuley et al., 2012). But recent advances in inhalation toxicology research highlight that electronic cigarettes are not a safer alternative for smokers (Arastoo et al., 2020; Farsalinos, 2018; Qasim et al., 2017). The main purpose of all of the tobacco products remains common, that is delivery of nicotine.

Cadmium (Cd) is one of the most hazardous inorganic elements with considerable toxic effects in humans, others include lead (Pb) mercury (Hg), and arsenic (As). In the general population, tobacco smoke remains one of the major sources of Cd exposure (Bernhoft, 2013). Occupational exposures and exposure through food, mainly in geographical areas with contaminated soil are also common. Worldwide smoking habits ensure a widespread exposure to chemicals released during the burning process of tobacco and contribute to issues related to human health and the environment. Tobacco smoke-associated toxicities are hard to attribute to any single chemical, either organic or inorganic, due to the complex nature of different phases (gaseous, volatile, and particulate) of the smoke. They are largely collectively attributed to tar, a viscous, dark brown mixture of chemicals released from tobacco during burning. However, several studies are available that reported toxicological effects of either selected individual chemicals found in tobacco smoke (Haussmann and Fariss, 2016) or the leachate from cigarette waste (Slaughter et al., 2011; Green et al., 2020).

The present investigation aims to study the toxicological interaction of Cd and nicotine in-vitro using A549 lung adenocarcinoma cells. The study is based on the hypothesis that Cd and nicotine may have additive or synergistic toxic effects in lung cells. For A549 cells' exposure to nicotine, a soluble salt of nicotine (nicotine hydrogen tartrate, NHT) was used in this investigation because of the need of the in-vitro aqueous conditions.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Advanced DMEM (Dulbecco's Modified Eagle Medium) (Gibco, Life Technologies Ltd., UK), nicotine hydrogen tartrate (BDH Chemicals Ltd., Poole, England), cadmium 1000 mg/L (Jobin Yvon SAS, Longjumeau, France), DMSO (VWR Chemicals, France), sodium chloride, potassium chloride, disodium hydrogen phosphate, and potassium dihydrogen phosphate were purchased from Scharlab S.L., Spain. Other consumables including culture flasks, tubes, and disposable pipettes were purchased from Corning, NY, USA.

### 2.2. Cell viability assay

Toxic effects of different concentrations of NHT, Cd, and NHT + Cd combination on A549 cell viability was assessed by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). Briefly, A549 cells, approximately 15,000 cells per well were seeded into 96-well plate in 100  $\mu$ L cell media. Seeded cells were incubated overnight at 37 °C and 5% CO<sub>2</sub> to allow them to adhere properly. Different concentrations of cadmium (Cd) and nicotine hydrogen tartrate (NHT) were added in different wells to see their cytotoxic effects and for comparison between them. Stock solutions of Cd and NHT were serially diluted in a 96 well plate using serum-free media. After several pilot experiments, a range of concentrations was selected for Cd and NHT for further investigation. The assay was conducted in triplicate. After 24 h. of exposure to Cd and NHT, 10  $\mu$ L of MTT solution (5 mg/ml PBS) was added in each well except the blank. The cells were incubated for 4 h to allow the metabolism of MTT by viable cells. Cell media was removed from all of the wells and 100  $\mu$ L of DMSO was added to dissolve the formazan of MTT. After 30 min. of incubation the plate was read at 570 nm using DMSO as blank.

Based on the MTT assay highly toxic and least toxic concentrations of NHT and Cd were selected for further investigation. The selected concentrations of NHT included 5 mM and 10 mM, whereas selected concentrations of Cd included 50  $\mu$ M and 100  $\mu$ M. A549 lung cancer cell lines were used for the investigation as both of the toxicants under investigation are known to be released with tobacco smoke and directly interact with lung tissue.

### 2.3. Apoptosis analysis

Apoptotic events were analyzed using a kit mainly containing AnnexinV-FITC and Propidium iodide (PI) (Invitrogen, Thermo Fisher Scientific, CA, USA) per the instructions provided with the kit. A549 cells were evaluated based on early apoptosis, late apoptosis, and necrotic death after 24 h exposure to different concentrations of Cd and NHT. Briefly, A549 cells ( $2.2 \times 10^5$  cells/well) were seeded into 6-well culture plates and incubated (37 °C and 5% CO<sub>2</sub>) to allow the cells to reach approx. 60% confluence. Then cells were exposed to Cd (50  $\mu$ M and 100  $\mu$ M), or NHT (5 mM and 10 mM), or NHT + Cd (5 mM + 50  $\mu$ M; 10 mM + 50  $\mu$ M; 5 mM + 100  $\mu$ M; 10 mM + 100  $\mu$ M) for 24 h. Cells were harvested using trypsin-EDTA (0.25%, Gibco) and washed twice with PBS. After removing supernatant PBS cells, 100  $\mu$ L Annexin V-binding buffer was added to resuspend the cells. In the next step, AnnexinV-FITC (5  $\mu$ L) and PI dye (1  $\mu$ L of the 150  $\mu$ M) were added and incubated for 15 min in the dark. At last, 400  $\mu$ L of 1X annexin-binding buffer was added. Unexposed cells were used as the negative control. Apoptotic vs necrotic cell populations were determined by flow cytometer (Cytomics FC 500; Beckman Coulter, CA, USA). Detection filters used include FL1 (Annexin V-FITC) and FL3 (Propidium Iodide). Data collection and analysis were performed using CXP-cytometer and CXP-Analysis Software V 3.0.

### 2.4. Reactive oxygen species evaluation

Total reactive oxygen species (ROS) generation in A549 cells after exposure to Cd and NHT was evaluated using ROS Assay Kit 520 (Invitrogen, Thermo Fisher Scientific, CA, USA). The assay is based on the deacetylation of DCFDA (2',7' -dichlorofluorescein diacetate) within the cells esterases to a non-fluorescent compound, which is later oxidized by ROS into fluorescent DCF (2', 7' -dichlorofluorescein). Cells were seeded and treated in the same way as explained above. Briefly, after 24 h exposure to Cd, NHT or combination cells were harvested and washed with PBS. Cell pellets were suspended in ROS assay buffer containing 1X ROS Assay stain. After 60 min. incubation (37 °C and 5% CO<sub>2</sub>) cells were analyzed by flow cytometer (Cytomics FC 500; Beckman Coulter, CA, USA). FL1 filter was used to detect the level of DCF fluorescence. Mean Fluorescence Intensities (MFI) of different cell exposure groups were compared with the unexposed cells (negative control).

### 2.5. Cell cycle analysis

To evaluate the effect of Cd and NHT on cell cycle events,  $5 \times 10^3$  A549 cells/well were seeded in a 24-well culture plate. Cells were incubated for 48 h (37 °C and 5% CO<sub>2</sub>) before exposing to Cd and NHT. After 24 h exposure cells were harvested and washed with cold PBS. Cell fixation was done using 70% ethanol (stored at -20 °C), and then stored at 4 °C for 2 h. For staining the DNA in fixed cells, 3  $\mu$ M PI concentration was used and cells were incubated with RNase A (100  $\mu$ g/mL) for RNA degradation. After 30 min incubation in dark, the samples were analyzed using a flow cytometer (Cytomics FC 500; Beckman Coulter, CA, USA) to obtain the DNA content data of cells reflecting cells in different cell

phases, using FL3 filter. Data collection and analysis were performed using CXP software V. 3. 0.

### 2.6. Statistical analysis

Data are presented as 'Mean ± SD'. The raw data were analyzed using GraphPad InStat (GraphPad Software Inc., La Jolla, CA). Data were subjected to One-way ANOVA followed by Tukey-Kramer Multiple Comparison Test to analyze statistical significance between different exposure groups.

## 3. Results

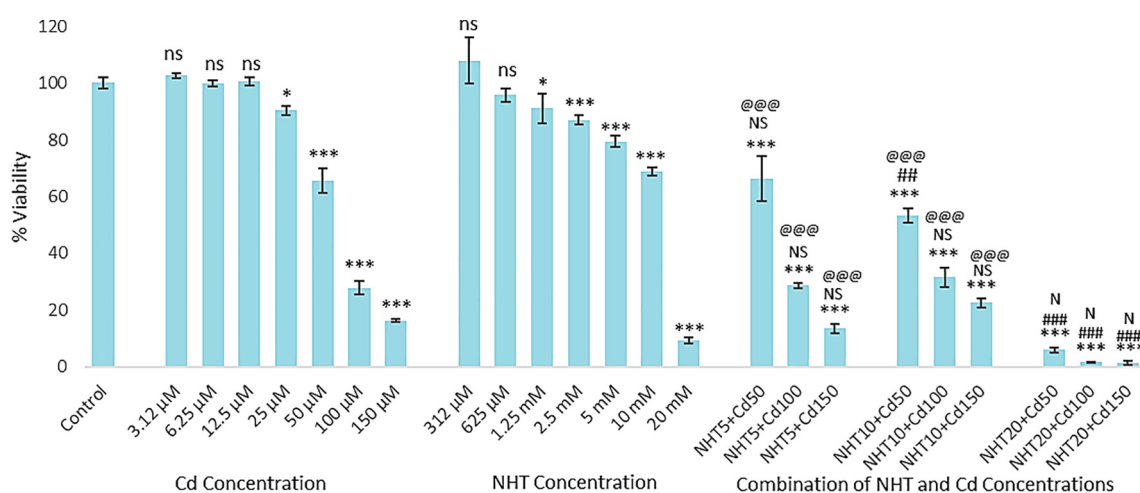
### 3.1. Effects of Cd and NHT on A549 cell viability

A549 cells were treated with NHT and Cd individually as well as in combination at different concentrations to determine their effects on the cell viability (Fig. 1). The effects were analyzed after 24 h of exposure by MTT. The results were compared with the control as well as between other treatment groups. The Cd concentration at 25 µM yielded substantial ( $p < 0.05$ ) effects while the effects on cell viability were significant ( $p < 0.001$ ) at three Cd concentrations i. e., 50 µM, 100 µM, and 150 µM in comparison to control. No significant effects were observed on cell viability at exposure to Cd concentrations i. e., 3.12 µM, 6.25 µM, and 12.5 µM when compared with control. It was observed that the cell viability decreased with increasing Cd concentration and the percentage of viable cells was highest at 3.12 µM and lowest at 150 µM. The  $IC_{50}$  of Cd was found to be 83 µM after 24 h of exposure. Exposure of cells to different NHT concentrations causes variable effects on their viability. The effects were non-significant at 312 µM, and 625 µM NHT concentrations, however, the effect on cell viability was significant ( $p < 0.05$ ) at 1.25 mM NHT concentration and more significant ( $p < 0.001$ ) at 2.5 mM, 5 mM, 10 mM, and 20 mM in comparison to control. The cell viability was seen decreasing with increasing NHT concentration and was highest at 312 µM and lowest at 20 mM. The  $IC_{50}$  of NHT was found to be 11.71 mM after 24 h of exposure. The combined effect of Cd + NHT exposure on cell viability was also measured and the combined treatment protocol was divided into 3 groups each group has 3 treatment combinations with constant NHT concentration but varying Cd concentration. The effect in the first combined-treatment group was non-

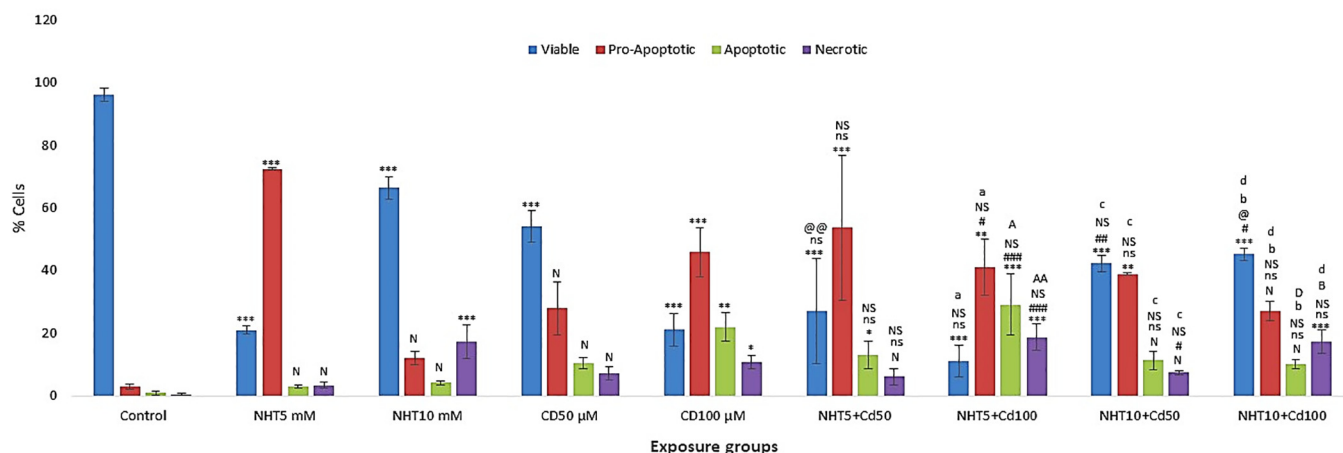
significant at all the three combination concentrations i. e., NHT5 + Cd50, NHT5 + Cd100, and NHT5 + Cd150 when compared with the same concentration group of Cd, but significant ( $p < 0.001$ ) when compared with control and the same concentration group of NHT. In this concentration group cell viability was highest at NHT5 + Cd50 and lowest at NHT5 + Cd150, demonstrating that while keeping NHT concentration constant and with increasing Cd concentration the percentage of viable cells decreases. The effect in the second combined-treatment group was significant ( $p < 0.01$ ) at NHT10 + Cd50 while non-significant at NHT10 + Cd100, and NHT10 + Cd150 when compared with the same concentration group of Cd, but highly significant ( $p < 0.001$ ) at all the three concentration when compared with control and the same concentration group of NHT. In the second combined treatment group, the NHT concentration was constant but double than the first group, however, different Cd concentrations were used but similar to the first combined treatment group. In this case, the cell viability was highest at NHT10 + Cd50 and lowest at NHT10 + Cd150. The last combination-treatment group involved three different Cd concentrations but similar to the first and second combined treatment groups. However, in this group, the NHT concentration was 20 mM. All the combined (NHT + Cd) concentrations i. e., NHT20 + Cd50, NHT20 + Cd100, NHT20 + Cd150 demonstrated non-significant effects on the cell line viability when compared with the same concentration group of NHT. However, all three combinations produced quite significant ( $p < 0.001$ ) effects on cell viability in comparison to control and the same concentration group of Cd. The percentage of viable cells was higher at NHT20 + Cd50 and lowest at NHT20 + Cd150.

### 3.2. Apoptotic effects of Cd and NHT in A549 cells

The treatment of A549 cells with different individual NHT and Cd as well as with their combination at different concentrations was done to determine their pro-apoptotic, apoptotic, and necrotic effects on the cell viability (Fig. 2a). The effects were analyzed after 24 h of exposure by flow cytometry (Fig. 2b). The results were compared with the control as well as between other treatment groups. The NHT concentration at 5 mM yielded significant ( $p < 0.001$ ) pro-apoptotic effects while apoptotic and necrotic effects were non-significant as compared to control. The NHT10 mM concentration produced significant ( $p < 0.001$ ) necrotic effects while pro-



**Fig. 1.** Effects of different concentrations of NHT, Cd and their combination on the viability of A549 cells analyzed by MTT after 24 h of exposure. ns = Not significant when compared with control; \* =  $P < 0.05$  when compared with control; \*\*\* =  $P < 0.001$  when compared with control; NS = not significant when compared with same concentration group of Cd; ## =  $P < 0.01$  when compared with the same concentration group of Cd; ### =  $P < 0.01$  when compared with the same concentration group of Cd; N = Not significant when compared with same concentration group of NHT; @@@ =  $P < 0.001$  when compared with same concentration group of NHT. NHT5 = NHT 5 mM; NHT10 = NHT 10 mM; NHT20 = NHT 20 mM; Cd50 = Cd 50 µM; Cd100 = Cd 100 µM; Cd150 = Cd 150 µM.



**Fig. 2a.** Effects of different concentrations of NHT, Cd and their combination on the apoptotic events in A549 cells analyzed by flowcytometry after 24 h of exposure.\* =  $P < 0.05$  when compared with control; \*\* =  $P < 0.01$  when compared with control; \*\*\* =  $P < 0.001$  when compared with control; N = not significant when compared with control; ns = not significant when compared with NHT treatment of same concentration; # =  $P < 0.05$  when compared with NHT treatment of same concentration; ## =  $P < 0.01$  when compared with NHT treatment of same concentration; ### =  $P < 0.001$  when compared with NHT treatment of same concentration; NS = not significant when compared with Cd treatment of same concentration; @ =  $P < 0.05$  when compared with Cd treatment of same concentration; a = not significant when compared with NHT5 + Cd50; A and AA =  $P < 0.05$  and  $P < 0.01$  respectively when compared with NHT5 + Cd50; b = not significant when compared with NHT10 + Cd50; c = not significant when compared with NHT5 + Cd50; d = not significant when compared with NHT5 + Cd100.

apoptotic and apoptotic effects were non-significant as compared to control. The treatment with Cd at the concentration of 50  $\mu\text{M}$  did not exhibit any significant increase in pro-apoptotic, apoptotic, or necrotic cells, however, the exposure decreased the number of viable cells significantly ( $p < 0.001$ ). The Cd concentration at 100  $\mu\text{M}$  yielded highly significant ( $p < 0.001$ ) pro-apoptotic effects while significant effects on apoptosis ( $p < 0.01$ ), and necrosis ( $p < 0.05$ ) were seen as compared to control. The cell group exposed to the combined NHT5 + Cd50 concentration demonstrated highly significant ( $p < 0.001$ ) pro-apoptotic effects and significant ( $p < 0.05$ ) apoptotic effects as well as non-significant necrotic effects when compared to the control group while depicted significant ( $p < 0.01$ ) effects on cell viability when compared with same Cd treatment concentration, however, pro-apoptotic, apoptotic, and necrotic effects were also non-significant when compared with Cd treatment of the same concentration (Cd 50  $\mu\text{M}$ ). The viability, pro-apoptotic, apoptotic, and necrotic effects were non-significant when compared with NHT treatment of the same concentration (5 mM). Similarly, the combined effect of NHT5 + Cd100 concentration demonstrated highly significant ( $p < 0.001$ ) apoptotic, and necrotic effects and significant ( $p < 0.01$ ) pro-apoptotic effects when compared to the control group. Also, apoptotic, and necrotic effects were highly significant ( $p < 0.001$ ), while pro-apoptotic effects were significant ( $p < 0.05$ ) when compared with the same NHT treatment concentration (5 mM). The viability, pro-apoptotic, apoptotic, and necrotic effects were found to be non-significant when compared with Cd treatment of the same concentration (Cd 100  $\mu\text{M}$ ). The effects on cell viability were also non-significant when compared with NHT treatment of the same concentration, while pro-apoptotic effects were also non-significant when compared with NHT5 + Cd50. Significant effects on apoptosis ( $p < 0.05$ ), and necrosis ( $p < 0.01$ ) were observed when compared with NHT5 + Cd50.

After 24 h exposure to NHT10 + Cd50 the effects on cell viability were highly significant ( $p < 0.001$ ), and the pro-apoptotic effects were significant ( $p < 0.01$ ) while apoptotic and necrotic effects were non-significant as compared to control. The viability and necrotic effects were significant ( $p < 0.01$ ,  $p < 0.5$ ) respectively when compared with NHT treatment of the same concentration (10 mM), pro-apoptotic and apoptotic effects remained non-significant. The viability, pro-apoptotic, apoptotic, and necrotic

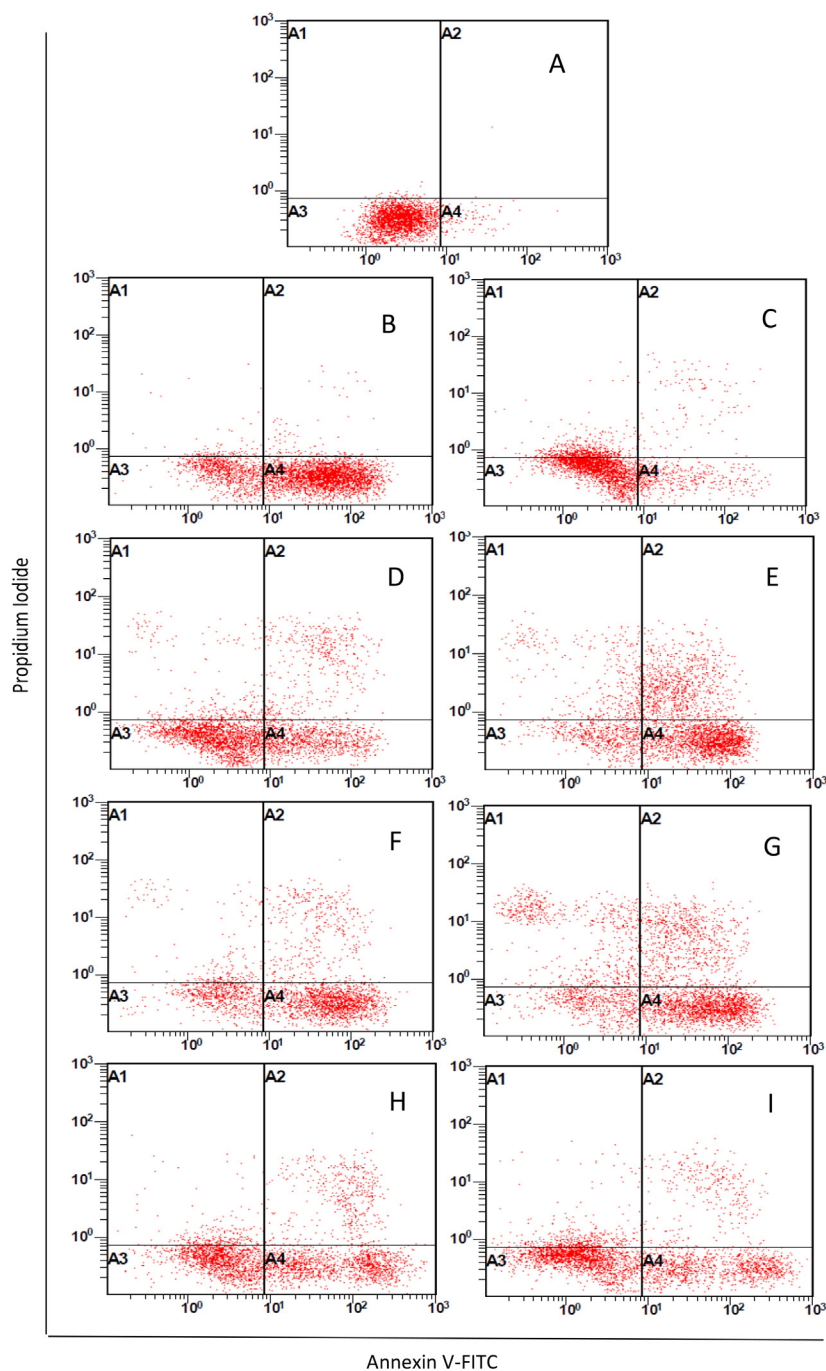
effects were non-significant when compared with Cd treatment of the same concentration (Cd 50  $\mu\text{M}$ ). Additionally, the viability, pro-apoptotic, apoptotic, and necrotic effects were non-significant when compared with NHT5 + Cd50.

The treatment with NHT10 + Cd100 had highly significant ( $p < 0.001$ ) effects on cell viability and necrosis while no significant pro-apoptotic and apoptotic effect was observed in comparison to the control group. The effects on cell viability were significant ( $p < 0.05$ ) while non-significant pro-apoptotic, apoptotic, and necrotic effects were observed when compared with NHT treatment of the same concentration (10 mM). Additionally, the viability effects were significant ( $p < 0.05$ ) whereas the pro-apoptotic, apoptotic, and necrotic effects were non-significant when compared with Cd treatment of the same concentration (Cd 100  $\mu\text{M}$ ). Non-significant effects were observed on cell viability, pro-apoptosis, apoptosis, and necrosis when compared with NHT10 + Cd50. Moreover, the viability, pro-apoptotic, and necrotic effects were non-significant while apoptotic effects were significantly lower ( $p < 0.05$ ) when compared with NHT5 + Cd100. The combination exposure that caused the most reduction in cell viability is NHT5 + Cd100 with the pattern of pro-apoptotic, apoptotic, and necrotic events appear to be similar to individual exposure to Cd 100  $\mu\text{M}$ .

### 3.3. Effects of Cd and NHT on cell cycle of A549 cells

The effect of NHT and Cd as well as that of their combination at different concentrations was determined on the cell cycle phases in A549 cells (Fig. 3a). The effects were analyzed after 24 h of exposure by flow cytometry using propidium iodide (Fig. 3b). The individual treatment with NHT5 mM and NHT10 mM demonstrated a significant ( $p < 0.001$ ) increase in subG1, and decrease in S, and G2-M cell populations while no significant effects were seen on the G1 phase as compared to the control group. Similarly, a highly significant ( $p < 0.001$ ) increase was observed in subG1, and a decrease in S, and G2-M population and significant ( $p < 0.05$ ) decrease were seen in the G1 phase with Cd50  $\mu\text{M}$  exposure as compared to control. These effects appear to be increased with increasing concentration to Cd100  $\mu\text{M}$  that exhibited highly significant ( $p < 0.001$ ) effects on all phases of cell cycle i.e., increased subG1 and decreased G1, S, and G2-M population.



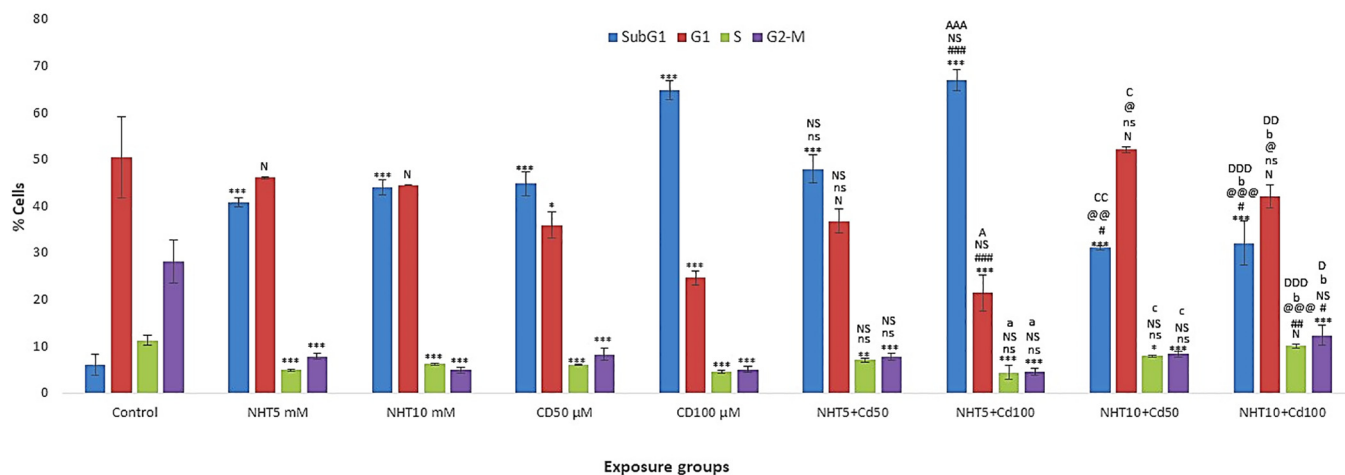


**Fig. 2b.** Effects of different concentrations of NHT, Cd and their combination on the apoptotic events in A549 cells analyzed by flowcytometry after 24 h of exposure. A: Control; B: NHT 5 mM; C: NHT 10 mM; D: Cd 50  $\mu$ M; E: Cd 100  $\mu$ M; F: NHT 5 mM + Cd 50  $\mu$ M; G: NHT 5 mM + Cd 100  $\mu$ M; H: NHT 10 mM + Cd 50  $\mu$ M; I: NHT 10 mM + Cd 100  $\mu$ M.

The combined treatment with NHT5 + Cd50 had highly significant ( $p < 0.001$ ) effects in terms of increasing subG1, and decreasing G2-M cell population, significant ( $p < 0.001$ ) effects on the S phase, and non-significant effects on G1 phase when compared with control. No significant differences were found when these effects were compared with the same individual concentrations of NHT (5 mM) and Cd (50  $\mu$ M). The NHT5 + Cd100 treatment combination induced significant ( $p < 0.001$ ) effects on all the four cell populations (subG1, G1, S, and G2-M) when compared with control. This combined treatment also demonstrated significant ( $p < 0.001$ ) effects on subG1, and G1 phases while non-significant effects on S, and G2-M phases, when compared with NHT treat-

ment of the same concentration, were observed. Also, non-significant effects were obtained on all the four phases due to this combined treatment when compared with Cd treatment of the same concentration (100  $\mu$ M). Moreover, NHT5 + Cd100 treatment induced significant effects on both ( $p < 0.001$ ) subG1, and ( $p < 0.05$ ) G1 phase but non-significant effects on S, and G2-M phases when compared with NHT5 + Cd50.

The effects of the NHT10 + Cd50 combination were highly significant ( $p < 0.001$ ) in terms of increase in subG1, and decrease in G2-M population, a significant decrease ( $p < 0.05$ ) in S phase population, and non-significant on G1 phase compared to control. However, this combined treatment had significant ( $p < 0.05$ )



**Fig. 3a.** Effects of different concentrations of NHT, Cd and their combination on the cell cycle phases in A549 cells analyzed by flowcytometry after 24 h of exposure. \* =  $P < 0.05$  when compared with control; \*\* =  $P < 0.01$  when compared with control; \*\*\* =  $P < 0.001$  when compared with control; N = not significant when compared with control; ns = not significant when compared with NHT treatment of same concentration; # =  $P < 0.05$  when compared with NHT treatment of same concentration; ## =  $P < 0.01$  when compared with NHT treatment of same concentration; ### =  $P < 0.001$  when compared with NHT treatment of same concentration; NS = not significant when compared with Cd treatment of same concentration; @ =  $P < 0.05$  when compared with Cd treatment of same concentration; @@ =  $P < 0.01$  when compared with Cd treatment of same concentration; @@@ =  $P < 0.001$  when compared with Cd treatment of same concentration; a = not significant when compared with NHT5 + Cd50; A and AAA =  $P < 0.05$  and  $P < 0.001$  respectively when compared with NHT5 + Cd50; b = not significant when compared with NHT5 + Cd50; c = not significant when compared with NHT5 + Cd50; CC =  $P < 0.01$  when compared with NHT5 + Cd50; d = not significant when compared with NHT5 + Cd100; DD and DDD =  $P < 0.01$  and  $P < 0.001$  respectively when compared with NHT5 + Cd100.

effects on the subG1 phase, and non-significant on all other three phases (G1, S, and G2-M) when compared with NHT treatment of the same concentration (10 mM). Moreover, when compared with Cd treatment of same concentration (50 μM), this combination caused significant ( $p < 0.001$ ,  $p < 0.05$ ) effects respectively on subG1, and G1 phases while non-significant effects on S, and G2-M phases. Finally, the combined effect of NHT10 + Cd50 was found significantly different ( $p < 0.01$ ) on subG1 and non-significant on all other three phases (G1, S, and G2-M) when compared with NHT5 + Cd50.

The treatment of cells with NHT10 + Cd100 induced a significant effect ( $p < 0.001$ ) on subG1, and G2-M phases while no significant effects were observed on G1, and S phases compared to control. This combination also demonstrated significantly different ( $p < 0.05$ ;  $p < 0.001$ ;  $p < 0.05$ ) effects respectively on subG1, S, and G2-M phases but non-significant on G1 phase when compared with NHT treatment of same concentration (10 mM). However, the effects were significantly different ( $p < 0.001$ ) on subG1, S phases, and G1 phase ( $p < 0.05$ ), and non-significant on G2-M phase when compared with Cd treatment of same concentration (100 μM). The effects were non-significant on all phases of the cell cycle when compared with NHT10 + Cd50. Lastly, this combined treatment group, nevertheless, induced highly significant ( $p < 0.001$ ) effects on subG1, and S phases while significant ( $p < 0.01$ ;  $p < 0.05$ ) effects respectively on G1, and G2-M phases when compared with NHT5 + Cd100.

### 3.4. ROS generation by Cd and NHT in A549 cells

The treatment of A549 cells with different individual NHT and Cd as well as with their combination at different concentrations was done to determine the ROS production (Fig. 4a). The effects on ROS generations were analyzed after 24 h of exposure by flow cytometry (Fig. 4b). The effects on ROS production were significant ( $p < 0.05$ ) at NHT5 mM, while highly significant ( $p < 0.001$ ) at the concentrations NHT10 mM, Cd50 μM, and Cd100 μM when compared with control. The exposure of A549 cell to NHT + Cd combination treatments such as NHT5 + Cd50, NHT5 + Cd100,

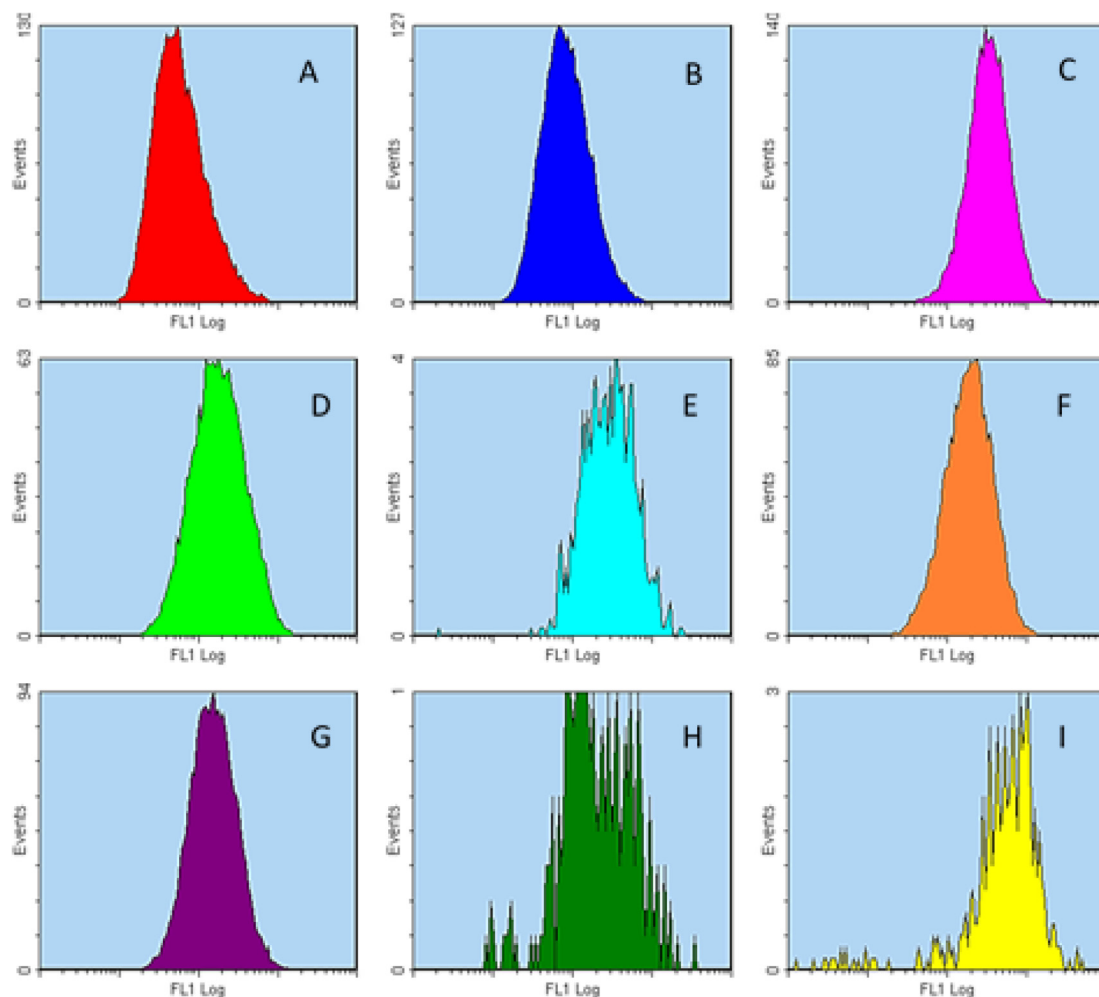
NHT10 + Cd50, and NHT10 + Cd100 yielded significant ( $p < 0.001$ ) ROS generation compared to control. The effects of NHT5 + Cd50 on ROS levels were non-significant when compared with Cd treatment of the same concentration (50 μM). Significant lower ( $p < 0.001$ ) effects on ROS production were observed at NHT5 + Cd100, and NHT10 + Cd50 concentrations when compared with individual Cd exposures. The difference of ROS generation by NHT5 + Cd100 was non-significant while the NHT10 + Cd50 combination produced different ( $p < 0.05$ ) levels of ROS compared to NHT5 + Cd50. Moreover, the effects on ROS generation were significantly higher ( $p < 0.001$ ) after NHT10 + Cd100 combination exposure when compared to NHT10 + Cd50, NHT5 + Cd50 and NHT5 + Cd100 combinations.

## 4. Discussion

Episodes of exposure to cadmium (Cd) and nicotine are not confined to tobacco smoking, it is also an environmental issue that arises with the release of cigarette waste and smoke into the environment (Qamar et al., 2020; Wu et al., 2016). Cadmium is a known carcinogen and chronic inhalation exposure through tobacco smoke cause elevated cadmium levels in the blood. Chronic exposure and high level of cadmium in blood actively reduce pulmonary functions in addition to other toxic effects including bronchogenic carcinoma, prostate cancer, pancreatic cancer, cervical cancer, peripheral arterial disease, cardiovascular diseases, obstructive lung disease, and various oral pathologies (Richter et al., 2017). Occupational exposure also adds to the levels of cadmium (Tavakkoli and Khanjani, 2016). Occupational exposure to cadmium has been reported to cause increased incidences of chromosomal aberrations and sister chromatid exchanges (Abraham et al., 2011). Cadmium levels in urine, after chronic exposures in cadmium workers, have been reported elsewhere to be correlated with neurobehavioral effects including psychomotor functions, peripheral neuropathy, and ability to concentrate, etc. (Viaene et al., 2000). Similarly, exposure to nicotine is ubiquitous.

However, such kinds of exposures are not limited only to cadmium and nicotine, when we talk about the smoking-associated





**Fig. 4b.** Effects of different concentrations of NHT, Cd and their combination on the ROS generation in A549 cells analyzed by flowcytometry after 24 h of exposure. A: Control; B: NHT 5 mM; C: NHT 10 mM; D: Cd 50  $\mu$ M; E: Cd 100  $\mu$ M; F: NHT 5 mM + Cd 50  $\mu$ M; G: NHT 5 mM + Cd 100  $\mu$ M; H: NHT 10 mM + Cd 50  $\mu$ M; I: NHT 10 mM + Cd 100  $\mu$ M.

of the toxicants in this investigation do not reflect toxic in-vivo concentrations after a real-life exposure event. Nevertheless, the experimental design and setup provided important information relevant to study toxicological interactions of NHT and Cd.

Results indicate concentration-dependent cytotoxic effects of individual exposures of NHT and Cd in A549 cells after 24 h. period reflected by reduction in cell proliferation. Nontoxic and highly toxic concentrations of NHT and Cd were dropped out of further investigation including apoptosis analysis, cell cycle, and ROS generation. In apoptosis analysis, it was found that a lower dose of NHT is inducing more proapoptotic/early apoptotic events than the higher dose that caused more necrotic cell death. 24 h cell cycle study reveals that the G2-M phase may be the target of higher concentrations of NHT. On the other side, Cd, lower and higher concentrations, appear to cause cell death mainly by apoptosis targeting cells in the G1 phase. These effects do not appear to alter much when the cells were exposed to the combination of NHT and Cd. Data indicate that the pattern of effects in apoptosis analysis, by NHT, is similar to what individual lower and higher concentrations exhibited mainly in terms of early apoptotic and necrotic events. However, Cd also appears to contribute to the pattern observed in apoptosis analysis. Overall, the combination of a lower concentration of NHT (5 mM) and the higher concentration of Cd (100  $\mu$ M) caused more toxicity than any other exposure in the present investigation. These effects were confirmed in cell cycle analysis.

NHT5 + Cd100 caused the highest number of cell accumulation in the SubG1 phase than any other exposure group. Based on the data analysis these effects may be attributed to the higher concentration of Cd than to NHT. They both have minimal toxic interaction probably due to their different mechanism of action. The mechanism of toxicity of Cd includes interference in DNA repair mechanism and apoptosis. At the cellular level, Cd is known to affect cell proliferation, differentiation, and induction of apoptosis (Rani et al., 2014). Cd can cause chromosomal aberrations and DNA mutations, mitochondrial damage, and inhibition of cellular respiration (Rafati Rahimzadeh et al., 2017; Patrick, 2003). However, these effects are dependent on the exposure time, for example in short-term exposure Cd causes apoptosis but in chronic exposure, it attenuates the apoptosis (Genchi et al., 2020). The latter, attenuation of apoptosis, contributes to carcinogenic effects of Cd. On the other side, most of the effects of nicotine are receptor-mediated through nicotinic acetylcholine receptors (nAChRs) (Arredondo et al., 2001; Arredondo et al., 2005; Arredondo et al., 2008; Arredondo et al., 2003). However, contribution to the generation of ROS remains common to Cd and nicotine (Barr et al., 2007; Malinska et al., 2019; Branca et al., 2020; Heyno et al., 2008; Lopez et al., 2006; Aspera-Werz et al., 2018).

All the exposures of NHT and Cd concentrations contributed to the generation of ROS during 24 h, however, that does not appear to contribute to any of the other parameters included in the pre-



sent investigation. In individual exposures, the higher concentration of NHT generated the highest levels of ROS, while the combination of high concentrations of NHT and Cd generated the highest levels of ROS.

NHT and Cd both are known toxicants and the main aim of the investigation was to focus on their toxic interaction in the A549 lung cell line. Individual toxic effects of NHT and Cd are confirmed in lung cells and a minimal toxic interaction of both is also observed. However, the study is limited to only 24 h exposure because a higher number of exposure groups and different time points are not feasible to conduct. Moreover, the 24 h exposure study did reveal minimal toxic interaction of NHT and Cd that did not encourage conducting the more complex and extended investigation. Also, the investigation is limited to endpoints focused on descriptive toxicology and did not focus on any probable mechanisms of toxicological interactions of NHT and Cd in the biological system. Still, the investigation successfully revealed minimal toxic interaction of NHT and Cd in A549 cells.

## 5. Conclusion

Exposure to tobacco smoke toxicants either through smoking or environmental tobacco smoke is a major health issue. The present investigation revealed toxic interactions of nicotine and cadmium in A549 lung cancer cells that remained minimal after a single exposure. However, individual toxicity levels remained unaltered and suggest that different constituents of tobacco smoke may interact minimally but contribute significantly to overall toxic effects, for example targeting different organs, cells, or cell organelles. This is the reason why the health effects of tobacco smoke are very complex and it remains a major preventable cause of mortality and morbidity in millions worldwide. Further investigations involving chronic exposures to Cd and nicotine are needed to reveal more on this issue.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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