

ORIGINAL ARTICLE

Deleterious effects of combination of lead and β -amyloid peptides in inducing apoptosis and altering cell cycle in human neuroblastoma cells

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ABSTRACT

Lead (Pb) is a toxic pollutant known to cause several abnormalities related to the brain, including cognitive dysfunction, and it is ubiquitous in nature. β -amyloid peptides (AP) are crucially involved in Alzheimer's disease (AD). It has been reported that there is a connection between lead and amyloid peptides in exerting similar kinds of altered functions in the brain and long-term exposure to lead leads ultimately to increased beta amyloid formation in the brain, lethal to human brain cells. There is still a lack of information on the mechanism by which Pb affects AP formation, exerting combined toxicity in AD patients. To fill the gap, we have systematically analyzed the toxicity individually and in combination of Pb and AP in human brain cells. We found that the combination of Pb and AP exerted a higher toxicity than individual exposures in human neuroblastoma cells. The lower inhibitory concentration values were determined by both time and concentration dependent manner on using MTT assay. The data resulted in the development of enhanced toxicity on exposure to Pb with both the combinations of AP(1-40) or (25-35) and with all combinations in human brain cells compared to individual exposures to Pb (1-40) or AP(25-35). The severe apoptotic effect and alteration in cell cycle by arresting at the S-phase evidenced the increased toxicity of combinational exposure to Pb and AP on human neuroblastoma cells. Furthermore, the quantitative determination of LDH and caspase-3 activity indicated the induction of severe toxicity. We conclude that both are synergistically associated with effects such as arresting the cell cycle and triggering apoptosis during the progression of Alzheimer's disease.

KEY WORDS: human neuroblastoma cells; lead (Pb); β -amyloid peptides (AP); apoptosis; cell cycle

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease associated with loss of memory, cognitive dysfunction, dementia and death. Research findings on animal models revealed that formation of extracellular deposits of amyloid plaques and intraneuronal fibrillary tangles are the causative agent of AD (Morishima-Kawashima & Ihara, 2002). The mechanism by which amyloid plaques cause the disease is still unknown. Several reports stated that increased toxic metal exposure was found to be lethal

to brain cells. Lead (Pb) is a systemic toxicant which affects vital organ systems, primarily the central nervous system in developing stages of the human brain. Thus children are at a greater risk than adults and chronic exposure even at very low concentrations affects the prefrontal cortex, hippocampus and cerebellum, which are responsible for planning and memory (Damstra, 1977; Hou *et al.*, 2012, Bihagi *et al.*, 2011). Due to its effect on the cerebellum, it leads to several neurological disorders such as dementia, cognitive dysfunction, schizophrenia, Parkinson's disease and Alzheimer's disease (Bakulski *et al.*, 2012).

Recent studies on animal models showed that rats injected β -amyloid peptides (1-40), (25-35) at the region of the cortex developed neurodegeneration, localized necrosis and lesions at the site of injection (Plant *et al.*, 2003). When studied in monkeys, the injections developed neurodegeneration (Maurice *et al.*, 1996) in the

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cerebellum and the signs closely resembled those found in AD (Basha *et al.*, 2005; Wu *et al.*, 2008). Many studies showed that APs readily form oligomers and larger aggregates and are toxic to cells (Sambamurthy *et al.*, 2011). AP(1-40) and (25-35) caused apoptosis and the production of robust free radicals and induced decreased viability (Miguel-Hidalgo & Cacabelos, 1998). But the mechanism by which it causes AD is still unknown. Research findings suggest that there is a link between Pb and AD and those exposed to Pb are more prone to develop AD in later stages. Since the intracellular effects of Pb and AP were similar, the combinational exposure to Pb and AP needs to be investigated. In the present study, we treated human neuroblastoma cells with Pb and AP(1-40) and (25-35) individually and in different combinations and analyzed the extent of toxicity in terms of apoptosis, lipid peroxidation levels and at different phases of the cell cycle. Our approach in investigating the mechanism of toxicity may provide roots in developing the target drugs to reduce the toxicity generated by both lead and amyloid peptides in the progression of Alzheimer disease.

Materials and methods

Materials

RPMI-1640 medium and oxaloacetate, pyruvate, and insulin (OPI) were obtained from Sigma. Fetal bovine serum, penicillin, streptomycin were procured from GIBCO. The kit used for the assay of apoptosis was purchased from Chemi-Con International, a serological company, (ApopNexin™ FITC Apoptosis detection kit #APT750). All other chemicals purchased from Sigma and Gibco were of analytical grade. Beta amyloid peptides (1-40) and (25-35) were purchased from Genscript Inc.

Preparation of lead and amyloid peptides

1000 μ M stock solution was prepared by dissolving appropriate amounts of lead acetate in DMSO. Amyloid peptides (1-40) and (25-35) were prepared by dissolution in sterile double distilled water and incubated for 24 hours at room temperature according to the instructions by Genscript.

Cell culture

SH-SY5Y Human neuroblastoma cells were grown in RPMI-1640 medium supplemented with 12% (v/v) fetal bovine serum, 50 μ g/ml penicillin-streptomycin and OPI (150 μ g/ml oxaloacetate, 50 μ g/ml pyruvate and 0.2 U/ml insulin) in a CO₂ incubator maintained at 37 °C with 5% CO₂ and 75% humidity. The medium was changed every three days and the cultures were passed at 80% confluence.

Trypan blue exclusion test of cell viability

Cell viability was assessed by Trypan blue dye exclusion: 0.25% Trypan blue solution was added to 10 μ l of pellet collected after trypsinization and the number of deeply stained cells, representing dead cells, was counted using a hemocytometer. The number of stained cells was

subtracted from the total count in order to determine the percentage of viable cells.

Exposure of cells to Pb and AP(1-40) & (25-35)

Cells were seeded at 2×10^4 cells per well in a 96-well plate and allowed to attach and grow for 2 days prior to treatment and were treated with varying concentrations of Pb acetate (0.01–10 μ M) in order to determine the IC₅₀ value. Simultaneously, cells were treated with 20–120 μ M and 1–20 μ M concentrations of AP(1-40) and (25-35) respectively to find their effect on cell growth. Further, cells were exposed to IC₅₀ concentrations of Pb and AP(1-40) and (25-35) individually and in combinations of these compounds for 24 hrs and cell viability was determined by MTT assay.

Cytotoxicity assay

Cytotoxicity was assessed by MTT [3-(4, 5 dimethylthiazol 2-yl) 2, 5-diphenyltetrazolium bromide] reduction assay. In brief, the cells 2×10^5 per well were seeded into 96 well culture plates and allowed to attach. The medium containing IC₅₀ concentrations of Pb (5 μ M), AP(1-40) (60 μ M) and AP(25-35) (8 μ M) was added to the cells individually and in combinations of Pb+AP(1-40), Pb+AP (25-35), AP(1-40)+AP(25-35) and Pb+AP (1-40)+AP(25-35) and incubated for 24 h. Then 10 μ l of MTT reagent was added to the culture and incubated in the dark for 4 h at 37 °C, followed by cell lysis by addition of 100 μ l of 0.4M acid-isopropanol. The plate was left at room temperature in the dark for 2 hrs and the relative amount of MTT reduction was determined based on the absorbance measured at 570 nm, using a plate reader.

Quantification of apoptosis

Early apoptosis and late apoptosis/necrosis of SH-SY5Y cells induced by Pb and AP(1-40) and (25-35) individually and in combinations were determined by annexin-V cell surface binding protein and analyzed by flow cytometry. Cells were seeded on 6-well plate and incubated for two days prior to the treatment with Pb, AP(1-40) and (25-35). After 48 hrs, they were treated with IC₅₀ concentrations of Pb and AP individually and in combinations like Pb (5 μ M), AP(1-40) (60 μ M), AP(25-35) (8 μ M), Pb+AP(1-40), Pb+AP(25-35), AP (1-40)+AP (25-35) and Pb+AP (1-40)+AP(25-35) for 24 hrs. Cells were washed twice with PBS, harvested, and resuspended in 200 μ l of binding buffer according to the manufacturer's instructions. To this cell suspension, 3 μ l of annexin V-FITC (a marker of early apoptosis) and 2 μ l of propidium iodide (a marker of late apoptosis) were added. After 15 min incubation, cell apoptosis was determined by flow cytometry (BD FACS Aria)

Determination of LDH

Cells, 2×10^5 per well, were seeded into 6 well culture plates and allowed to attach. The medium containing IC₅₀ concentrations of Pb (5 μ M), AP(1-40) (60 μ M) and AP(25-35) (8 μ M) was added to the cells individually and in combinations of Pb+AP(1-40), Pb+AP(25-35),

β -AP(1-40)+AP(25-35) and Pb+AP(1-40)+AP(25-35) and incubated for 24 hrs. Cell lysates were prepared according to the manufacturer's protocol and by adding 20 μ l of TritonX-100 and 20 μ l of assay buffer to the pellet and incubated at room temperature for 1 hr. To the 100 μ l of supernatant 100 μ l of reaction solution was added and incubated at room temperature for one hour on an orbital shaker. The absorbance was read at 490 nm with a plate reader.

Determination of Caspase-3

Different combinations of Pb and AP(1-40) and (25-35) exposed SH-SY5Y cells were used to prepare cell lysates according to the manufacturer's protocol. To the prepared cell lysate, 30 μ l of dilution buffer and 50 μ l of 2X reaction buffer of 10mM DTT were added. Then 5 μ l of 4mM DEVD-pNA substrate was added and incubated for 1–2 hrs at room temperature. The absorbance was read at 400 nm.

Determination of cell cycle analysis by flow cytometer

Cells were seeded in 6-well plates and grown for two days prior to the treatment with Pb and AP with respective IC₅₀ concentrations of Pb and AP(1-40) and (25-35) individually and in combinations for 24 hrs. After 24-hr treatment, cells were collected and centrifuged at 1500 rpm for 5 min. Then the pellet was collected and washed 3 times with 1X PBS and fixed in 70% ethanol for 48 hrs. After fixation, it was centrifuged at 1500 rpm and 100 μ g/ml RNase and 50 μ g/ml propidium iodide were added to the pellet and incubated for 1 hour at room temperature in dark. Cell cycle analysis was determined by flow cytometer (BD FACS ARIA).

Statistical analysis

Data were expressed as mean \pm SD of at least four determinations from each group, repeated at least five times on different occasions. Statistical analysis was performed using one-way analysis of variance (ANOVA) and the statistical significance was assumed at $p < 0.05$.

Results

Initially, Human SH-SY5Y neuroblastoma cells were exposed to Pb with a broad range of concentrations from 1000 μ M to 0.0001 μ M for 24 hrs. The viability was decreased from 100% to 10% in a concentration dependent manner. Further, to assess lower levels of inhibitory concentrations, the cells were exposed to 1–10 μ M of Pb which resulted in a significant decrease in viability from 93.3% to 34% for 24 hrs and a 50% decrease (IC₅₀) in viability was observed at 5 μ M. A similar concentration dependent decrease in viability was observed when exposed to different time intervals of 12 hrs, 24 hrs and 48 hrs. Amyloid peptide (25-35) exposure with different concentrations ranging from 20 μ M to 2 μ M on SH-SY5Y cell lines showed a decrease in viability from 91% to 7.3%. A significant 50% decrease (IC₅₀) in viability was observed

at 8 μ M concentration. A similar concentration dependent decrease in viability was observed when exposed for 24 hrs, 48 hrs and 72 hrs. Similarly, when AP(1-40) with different concentrations from 120 μ M to 20 μ M was exposed to cells for 24 hrs a significant decrease in viability from 92.6% to 14.1% resulted. A 50% decrease in viability was observed at 60 μ M concentration. A similar concentration dependent decrease in viability was observed when exposed for different time intervals of 12 hrs, 24 hrs and 48 hrs. Exposure to the combination of Pb and AP for 48 hrs showed 88.4% decrease in viability (Figure 1).

Treatment with the combination of Pb and AP resulted in the induction of apoptosis in individually exposed cells. On analysis, the percentage of apoptotic cells was for Pb 3.9%, AP(1-40) 12.8%, AP(25-35) 0.7% and in combinations such as Pb+AP(1-40) it was 22.2% Pb+AP(25-35) 9.8% AP(1-40)+AP(25-35) 2.4% and the combination of all, Pb+AP(1-40)+AP(25-35) as 24.9%. From the flow cytometry data it is clearly evident that Pb and AP were showing increased levels of apoptosis in cells when exposed with Pb in combination of AP as 42% (apoptosis + necrosis) than individual exposure with Pb (32.5%) or AP(1-40) (24%) or (25-35) (8.8%) (Figure 2).

LDH is a cytosolic enzyme which gets released into the culture media when cytolysis or plasma membrane damage occurred and its quantitative estimation reveals the amount of toxicity of the treated compounds on the brain cells. In the present study, on exposure to the combinations of Pb with AP(1-40) and (25-35) led to elevated levels of LDH as 56% when compared with individual exposures of Pb, AP(1-40), AP(25-35) as 25%, 26%, 19% LDH levels respectively (Figure 3).

Caspase-3 is a marker of cellular apoptosis. Caspase-3 activity was more than 21.7% when used in combinations of both Pb and AP(1-40) and (25-35). Pb with either of the combinations of AP(1-40) or AP(25-35) was showing increased levels of caspase-3 activity as 10% and 4% when compared with individual exposures of Pb, AP(1-40), AP(25-35) as 3%, 2.3%, 2% respectively (Figure 4).

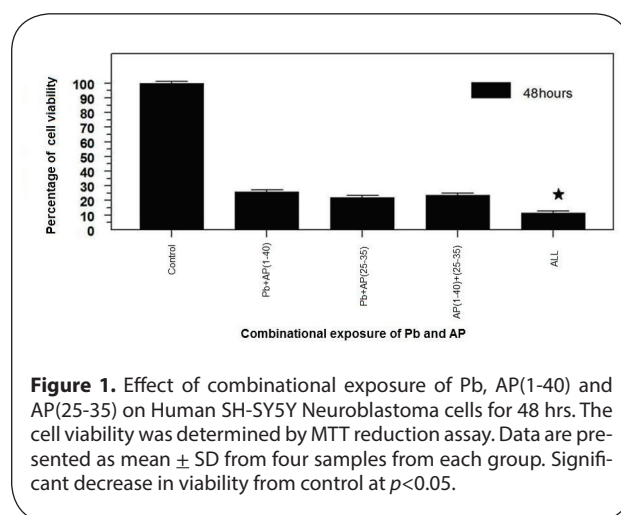


Figure 1. Effect of combinational exposure of Pb, AP(1-40) and AP(25-35) on Human SH-SY5Y Neuroblastoma cells for 48 hrs. The cell viability was determined by MTT reduction assay. Data are presented as mean \pm SD from four samples from each group. Significant decrease in viability from control at $p < 0.05$.

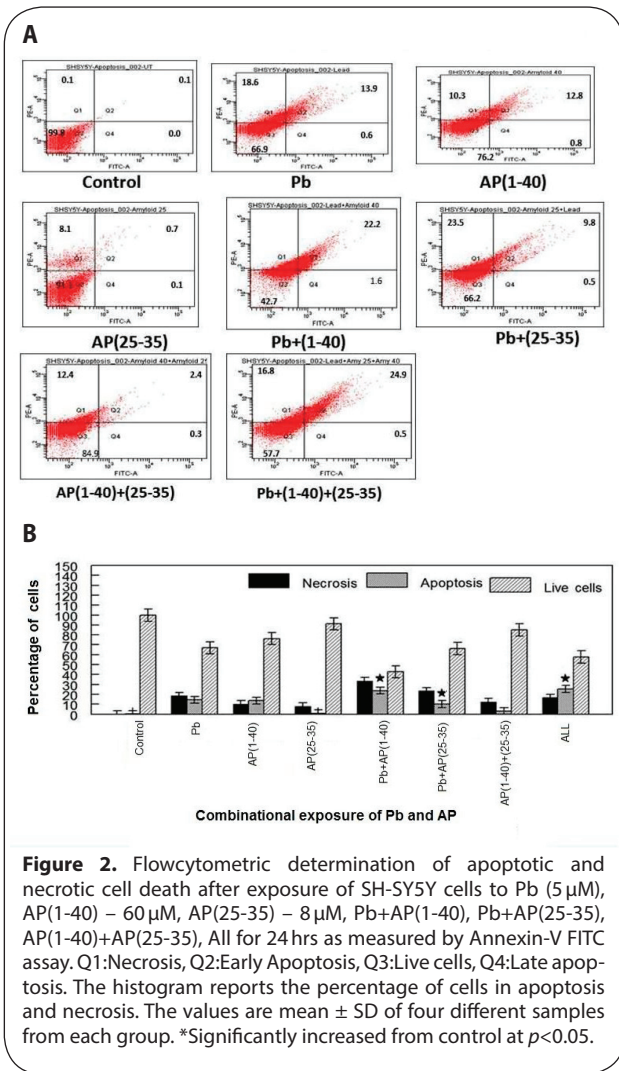


Figure 2. Flow cytometric determination of apoptotic and necrotic cell death after exposure of SH-SY5Y cells to Pb (5 μM), AP(1-40) – 60 μM, AP(25-35) – 8 μM, Pb+AP(1-40), Pb+AP(25-35), AP(1-40)+AP(25-35), All for 24 hrs as measured by Annexin-V FITC assay. Q1:Necrosis, Q2:Early Apoptosis, Q3:Live cells, Q4:Late apoptosis. The histogram reports the percentage of cells in apoptosis and necrosis. The values are mean ± SD of four different samples from each group. *Significantly increased from control at $p < 0.05$.

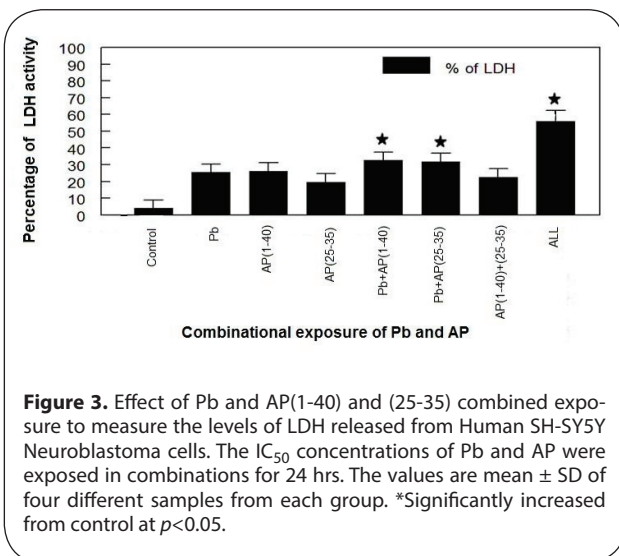


Figure 3. Effect of Pb and AP(1-40) and (25-35) combined exposure to measure the levels of LDH released from Human SH-SY5Y Neuroblastoma cells. The IC₅₀ concentrations of Pb and AP were exposed in combinations for 24 hrs. The values are mean ± SD of four different samples from each group. *Significantly increased from control at $p < 0.05$.

Cell cycle analysis was determined by flow cytometry and the treatment with the combination of Pb and AP arrested cell cycle at S-phase in cells. PI staining represents the amount of DNA present in the histograms

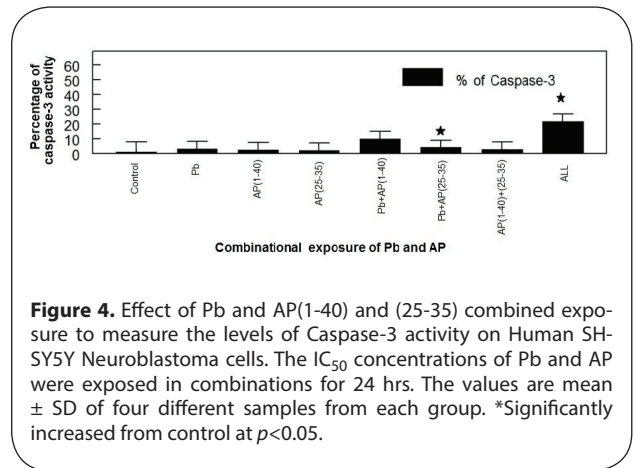


Figure 4. Effect of Pb and AP(1-40) and (25-35) combined exposure to measure the levels of Caspase-3 activity on Human SH-SY5Y Neuroblastoma cells. The IC₅₀ concentrations of Pb and AP were exposed in combinations for 24 hrs. The values are mean ± SD of four different samples from each group. *Significantly increased from control at $p < 0.05$.

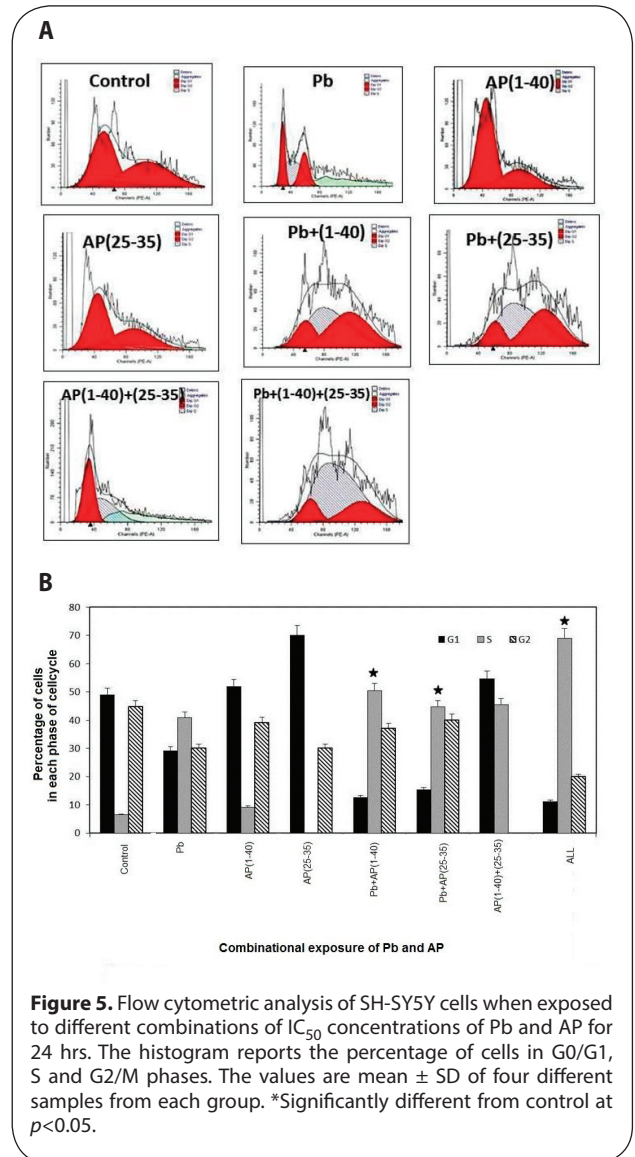


Figure 5. Flow cytometric analysis of SH-SY5Y cells when exposed to different combinations of IC₅₀ concentrations of Pb and AP for 24 hrs. The histogram reports the percentage of cells in G0/G1, S and G2/M phases. The values are mean ± SD of four different samples from each group. *Significantly different from control at $p < 0.05$.

analyzed from cell cycle analysis of flow cytometer. The following results were obtained when treated with Pb – 40.8%, AP(1-40) – 9.18%, AP(25-35) – 0.2% individually, whereas in combinations Pb+AP(1-40) – 50.3%,

Pb+AP (25-35) – 44.6%, AP (1-40)+AP (25-35) – 45.4% and when treated with all a 68% arrest in the S-phase was observed (Figure 5).

Discussion

Several reports have established the lethal effects of Pb and AP(1-40) individually in rat models (Takuwa *et al.*, 1995; Clementi *et al.*, 2005) and also reported that AP formation and Pb exposure are interlinked in various neurological disorders, and specifically in Alzheimer's disease. Still the connection between them is imprecise from a mechanistic standpoint. The current work is mainly devoted to answer two questions: i) how are the AP formation and Pb linked to each other? ii) What is their role in increasing lethality to human brain cells? We tried to address these fundamental questions by analyzing the lethal pathways of the cell, namely apoptosis and necrosis. From the analysis we conclude that not the individual but the combined exposure is highly lethal to SH-SY5Y cells, which are capable of differentiating into neuronal cells (Abemayor & Sidell, 1989), and are generally considered a model for Alzheimer's and Parkinson's disease (Sheehan *et al.*, 1997; Swettenham *et al.*, 2005; Lambert *et al.*, 1994; Garcia-Gill *et al.*, 2003). The combinational exposure of Pb and AP on SH-SY5Y resulted in significant increase in the production of free radicals and decrease in viability showing their toxic effect on SH-SY5Y cells, confirming that the combination has its severe toxic effect. To confirm and investigate further the level of apoptosis that is being induced, the amount of Caspase-3 and LDH release and cell cycle analysis were performed.

We conclude that Pb exposure leads to activation of caspase-3, marker of cellular apoptosis. Elevated expression of caspase-3 indicates the greater apoptotic effect of combinational exposure of Pb with either of the AP, compared to individual exposure. Further to substantiate the above finding we performed apoptosis assay using Annexin-V staining, clearly indicating that the combination of Pb and AP leads to necrosis and apoptosis rather than the individual exposure at very low concentrations.

In our experiments, data indicated that SH-SY5Y cell population when treated with Pb, AP(1-40), AP(25-35) showed a significant decrease at G2 phase and increase in cell population at S-phase, whereas the combination of Pb and AP showed severe decrease in G2 phase. We observed that while the G2 population of SH-SY5Y when treated with Pb and AP was slightly decreased and S-phase population was increased, the combination of Pb+AP (1-40), Pb+AP (25-35) or Pb+AP (1-40)+AP (25-35) showed the increased toxic effect at G1 phase. Also, it is evident that SH-SY5Y on exposure to the combination of Pb and AP(1-40) and (25-35) has severe inhibitory effect on G1 population by halting at G2/M phase. G2/M phase is the checkpoint where cells are allowed to repair the damaged DNA. Blocking at G2/M checkpoint reduces the possibility to repair and leads to apoptosis or necrosis. We observed that the mechanisms involved from S-phase to

G2/M phase may be the targets for using any therapeutic approach to reduce the combined toxicity of Pb and AP.

In conclusion, the combinations of Pb and AP when exposed to brain cell lines resulted in arresting the cell cycle at S-phase and G2 phases, from which it is evident that the combinational exposure arrested the proliferative phase and growth phase of the cell cycle. With the results obtained from our study, it was observed that, compared to individual exposures, the combinational exposure of Pb and AP was highly toxic as it inhibited cellular proliferation, induced oxidative stress, caused apoptosis and altered the cell cycle at cellular level. This approach provides the possibility to evaluate the genes which are involved in altering S-phase to G2/M-phases and further to identify therapeutic approaches to reduce the toxicity generated by both Pb and AP.

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Conflict of interest: All the authors declare that they do not have any conflict of interest.

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