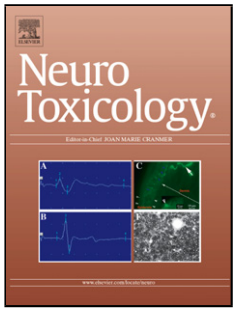

Title: Particulate matter cytotoxicity in cultured SH-SY5Y cells is modulated by Simvastatin: toxicological assessment for oxidative damage

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Particulate matter cytotoxicity in cultured SH-SY5Y cells is modulated by Simvastatin: toxicological assessment for oxidative damage

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Highlights

- There's a correlation between environmental particulate matter and health effects.
- We studied *in vitro* simvastatin's potential protective effect on ROFA cytotoxicity.
- ROFA decreased cell viability, increased ROS generation, apoptosis and senescence.
- Pretreatment with simvastatin attenuated the adverse effects observed.
- Simvastatin may provide protection against particulate matter-induced neurotoxicity.

Abstract

Epidemiological studies have shown a positive correlation between environmental particulate matter and adverse health effects. In particular, Residual Oil Fly Ash (ROFA) induces inflammation and reactive oxygen species (ROS), exerting not only local, but also systemic adverse effects. Previously, in an experimental animal model, we found that Simvastatin (Sv) pretreatment was effective in preventing ROFA induced lung inflammation. Herein, using the human neuroblastoma SH-SY5Y cell line as a neurotoxicity *in vitro* model, we studied the potential Sv protective effect on ROFA cytotoxicity. We evaluated cell viability by the MTT assay, superoxide anion generation by NBT test, Nrf2 activation by immunofluorescence, apoptosis by cleaved-PARP and active-caspase 3 expressions, and senescence by β–galactosidase activity. SH-SY5Y cells exposed to ROFA (10 and 50 µg/ml) for 24 hs showed decreased cell viability, increased superoxide anion generation, apoptosis and senescence. Pretreatment with Sv (1 µM) for 6 days, restored cell viability to basal levels, reduced ROFA-induced O_2^- generation as well as the number of apoptotic and senescent cells. Sv pre-treatment stimulated the basal and ROFA-induced levels of Nrf2 nuclear translocation suggesting that activation of the cellular antioxidant defense system prevented particle-induced oxidative stress. In parallel, rescue experiments with mevalonate did not modify the effects of SV pretreatment in any of the parameters evaluated in this study. We conclude that simvastatin may provide neuroprotection against air particulate matter-induced neurotoxicity independently of its ability to inhibit cholesterol synthesis.

Keyword: SH-SY5Y cells; simvastatin; cytotoxicity; ROFA; ambient particle; air pollution

Introduction

Epidemiological studies have shown a positive correlation between decreased air quality levels and adverse health effects (Brunekreef and Holgate, 2002). Anthropogenic emissions are the main contributors to environmental particulate matter (PM) burden and consist mainly of motor vehicle emissions and fossil fuel combustion during power generation and industrial processes (Nel, 2005). Among this last category, Residual Oil Fly Ash (ROFA) results from an incomplete combustion of carbonaceous materials and significantly contribute to ambient air PM burden (Costa and Dreher, 1997). In fact, inhaled or instilled ROFA induces *in vivo* a typical inflammatory response in the respiratory tract defined by the recruitment of polymorphonuclear cells (mainly neutrophils) into the lung parenchyma, a rise in the mucin PAS-positive cells of the respiratory epithelium and the release of proinflammatory cytokines like IL-1, IL-6, IL-8 and TNFα (Antonini et al., 2004; Costa et al., 2006). Moreover, ROFA not only exerts an inflammatory response but, an imbalance on the oxidative metabolism due to its high transition metal content (namely iron, nickel and vanadium) through the generation of reactive oxygen species (ROS) (Ghio et al., 2002).

In normal cells, ROS are produced in a controlled manner regulating different cell processes (cell division, inflammation, immune function, autophagy, and stress response). Upon ROS generation, cytoplasmic Nrf2 is released from its inhibitor Keap-1, translocates into the nucleus, and forms a cofactor complex that binds to specific antioxidant responsive elements (ARE) found in promoters of phase II antioxidant and detoxifying enzymes (Kensler et al., 2007). On the contrary, uncontrolled production of ROS, consequence of xenobiotics like PM, results in oxidative stress that if not counterbalanced by the antioxidant defense system to maintain the redox homeostasis in the cell, impairs cellular functions and may lead to cytotoxicity and cell death (Ma, 2013).

Strong scientific evidence supports that the deposition of small particles in the lung provokes not only local, but also systemic adverse effects, when translocate from the lung to the systemic circulation (Brook and Rajagopalan, 2009; Kampfrath et al., 2011; Ying et al., 2009). In particular, the neuropathological effects of PM were first described in mongrel dogs

where chronic brain inflammation and an acceleration of Alzheimer's disease-like pathology were evidenced by histology, suggesting that the brain is also adversely affected by air pollutants (Calderón-Garcidueñas et al., 2002; Calderón-Garcidueñas et al., 2003). Later, it was found that air pollution in humans is associated to cerebral hemodynamics and dangerous effects on structural brain aging, even in dementia and stroke-free individuals (Wellenius et al., 2013; Wilker et al., 2015).

On the other hand, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors (statins) that are the most effective treatment for hypercholesterolemia and management of coronary heart disease (Law et al., 2003; Tobert, 2003) are, albeit with some controversies, used in the treatment of neurodegenerative diseases or conditions such as cerebrovascular disease (Goldstein et al., 2008; Nassief and Marsh, 2008), Alzheimer disease (Eckert et al., 2005) and Parkinson's disease (Friedman et al., 2013; Lee et al., 2013; Wahner et al., 2008) among other illnesses. The underlying molecular mechanisms of the beneficial effects of statins for these diseases are poorly understood and probably independent of their ability to inhibit the HMG-CoA reductase.

We have previously shown that Simvastatin (Sv) pretreatment in both ROFA and in Urban Air Particles from Buenos Aires (UAP-BA) exposed animals, reduced lung oxidative stress and inflammation, therefore exerting a protective effect against environmental pollution (Ferraro et al., 2011).

Simvastatin, among other statins, due to its characteristics which includes the ability to cross the blood brain barrier is considered the most appropriate to prevent neurodegenerative conditions (Sierra et al., 2011). In this context, we sought to elucidate the *in vitro* effect of Sv on human neuroblastoma SH-SY5Y cell line exposed to ROFA, assessing cell viability, oxidative metabolism, apoptosis and senescence.

Materials and Methods

Ambient Air Particle Suspension

Residual Oil Fly Ash (ROFA) particles were collected from Boston Edison Co., Mystic Power Plant (Everett, MA, US) and were kindly provided by Dr. J. Godleski (Harvard School of Public Health, MA, US). ROFA samples from this source have been previously characterized in terms of elemental composition and particle size (Killingsworth et al., 1997; Ferraro et al., 2011). Vanadium, nickel and iron are the predominant metals present as water-soluble sulfates, and particle mean aerodynamic diameter is 2.06±1.57 μm. PM samples were freshly prepared by suspending ROFA particles in sterile saline solution (0.5 mg/ml), followed by 10 min incubation in an ultrasonic water bath before use.

Human Neuroblastoma SH-SY5Y Cell Culture and Simvastatin or Mevalonate Pre-Treatment

SH-SY5Y human neuroblastoma cells were purchased from American Type Culture Collection (ATCC®, Manassas, VA). Cells were plated in 75-cm^2 tissue culture flasks (BD Falcon, San Jose, CA) and grown in a 1:1 mixture of EMEMα (Invitrogen Corp**.,** Carlsbad CA) and F-12 Nutrient Mixture (SIGMA-Aldrich, St. Louis, MO**)** supplemented with 10% FBS and 1% penicillin–streptomycin (100 I.U./ml penicillin and 100 μg/ml streptomycin) (Gibco, Grand Island, NY)**.** Neuroblastoma cells were maintained in 5% CO_2 at 37ºC. For all experiments cells were plated initially at a density of 160,000 cells/ml.

Neuroblastoma cells in culture were treated during 6 days with 1 μM Simvastatin (Sv) (Sigma-Aldrich, Cat #S6196, St. Louis, MO) and/or 1 mM Mevalonate (Mv) (Sigma-Aldrich, Cat #41288, St. Louis, MO).

Stock solutions of Simvastatin (Sv, 0.1 M) and Mevalonate (Mv, 1M) were prepared in ethanol:NaOH (95%) and phosphate buffer saline (PBS, pH = 7.2) respectively, and stored in aliquots at -20ºC until use. Sv concentrations were performed in supplemented media as previously described (Butterick et al., 2010; Johnson-Anuna et al., 2007). Medium was

changed every other day during the growth and treatment phases, and both Sv and Mv were added fresh to the media during each medium change.

Ambient Air Particle Protocol Exposure

SH-SY5Y cells were cultured in 24 well plates in the presence or absence of Sv, or Sv + Mv and then exposed to ROFA (5, 10 or 50 µg/ml) for 24 hs. After exposure, cell cultures were assayed for cell viability, superoxide anion generation, apoptosis and senescence. It is worth to note that in order to ensure a uniform distribution, ROFA particles were sonicated for 10 min before use.

Cell Viability: MTT Assay

Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, Cat #M5655) assay, as described elsewhere (Molinari et al., 2003; Morgan, 1998). This assay is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases of living cells**.** Briefly, media from controls or exposed to ROFA particles were removed, cell cultures were washed twice with PBS 1X, and 0.5 ml fresh complete growth medium supplemented with 50 µl MTT (4 mg/ml in PBS) was added for 3 hs. Immediately after incubation, 250 µl/well of SDS (10%) was added to stop the MTT reaction and solubilize the formazan precipitate. The optical density (OD) of the final solution was measured 24 hs later spectrophotometrically at 570 nm (Shimadzu UV-1201V).

Superoxide Anion Generation: NBT Assay

Superoxide anion (O_2^-) was evaluated on SH-SY5Y cultures by the Nitro Blue Tetrazolium (NBT) assay (Sigma-Aldrich, Cat #N6876) according to Segal AW (Segal, 1974). Briefly, 1ml NBT (0.1% in PBS) was added to each well and incubated for 45 min at 37ºC. After incubation, paraformaldehyde (0.4%) was added to stop the reaction and fix the cells. The presence of a blue formazan precipitate was indicative of reactive cells. At least 200

cells (reactive and non reactive) were counted by light microscopy (Nikon Alphaphot-2 V52) as described elsewhere (Molinari et al., 2000). Results are expressed as the percentage of reactive cells.

Cellular senescence: β–galactosidase assay

Senescent SH-SY5Y cells were histochemically detected by the Senescence β–galactosidase Staining kit (Cat # 9860, Cell Signaling Technology, Danvers, MA) following manufacturer's instruction. At least 200 cells per well were counted by light microscopy (Nikon Alphaphot-2 V52). Results are expressed as the percentage of positive stained cells.

Caspase-3 activity, PARP and Nrf2 Immunocytochemical Determination

Caspase-3 and Poly ADP ribose polymerase (PARP) were determined using Anti-Active Caspase-3 pAB (Promega, Cat.Nº: G748, Madison, WI) and Anti-PARP p85 Fragment pAB (Promega, Cat.Nº: G7341) antibodies. Nrf2 transcription factor was assayed using anti-Nrf2 (Santa Cruz Biotechnology, Cat.Nº: sc-722, Dallas, TX). Briefly, cells were fixed with paraformaldehyde (4%) and permeabilized with Triton X-100 (0.2%). The fixed cells were blocked with Bovine Serum Albumin (BSA) (1%), and incubated with the primary antibody for Caspase-3 (1:500), PARP (1:250), or Nuclear factor erythroid 2-related factor 2 (Nrf2) (1:250) diluted in BSA (1%) during 18 hs. Cells were washed with PBS and incubated with secondary antibody conjugated with Cy-3 (1:100) for 1 hs. In order to identify cell nuclei, SH-SY5Y cells were counterstained with 100 nM DAPI (Sigma-Aldrich, Cat #D9542, St. Louis, MO) for 5 min. Cells were observed under a Nikon TE300 fluorescence microscope (λem = 615 nm for Cy3 and λem = 460 nm for DAPI). Results are expressed as the percentage of positive stained cells for Caspase 3, PARP or nuclear Nrf2.

Statistical analysis

All endpoints were run in triplicates for each experimental condition. One-way ANOVA with Newman-Keuls multiple comparisons post test was performed using GraphPad Prism

version 5.04 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

Results

Simvastatin prevents viability decrease on ROFA exposed SH-SY5Y cells

To determine if ROFA was toxic on SH-SY5Y cultures, cells were exposed to ROFA (5, 10 and 50 µg/ml) for 24 hs. At the lowest concentration employed, no changes were observed on cell viability using the MTT assay whereas, a significant (p<0.001) reduction on cell viability occurred in response to both 10 and 50 µg/ml of ROFA, suggesting a dose dependent toxicity in this neuroblastoma cell line (Figure 1).

We also evaluated the ability of simvastatin (Sv) to attenuate the toxic effect of ROFA on SH-SY5Y cells. Cells were pre-treated with Sv (1 µM) for 6 days and then exposed to ROFA (5, 10 and 50 µg/ml) for 24 hs as described under Materials and Methods. Sv pre-treatment attenuated ROFA toxic effects restoring cell viabiliy to basal levels (Figure 1). This effect was independent of its ability to inhibit HMG-CoA, since co-incubation with mevalonate (Mv) (by product of HMG-CoA reductase) did not modify Sv protective effect (Figure 1).

In order to assay whether cell viability is modified by Sv or Mv pre-treatment, SH-SY5Y cells were incubated with Sv: 0.1, 1 and 10 µM or Mv: 0.1, 1 and 2 mM for 6 days. Neither Sv nor Mv altered SH-SY5Y viability, evaluated by MTT assay, at the concentrations doses employed (C = 0.719±0.033; $Sv_{0.1}$ = 0.670±0.011; Sv_1 = 0.651±0.005; Sv_{10} = 0.668±0.011; $Mv_{0.1}$ = 0.701±0.004; Mv_1 = 0.682±0.016; Mv_2 = 0.672±0.017).

Simvastatin attenuates ROFA adverse effect on superoxide anion generation

Based on the known oxidative stress-inducing properties of PM, we evaluated the generation of superoxide anion (O_2^-) on SH-SY5Y cell cultures exposed to ROFA (5, 10 and 50 µg/ml) for 24 hs. Microphotographs of exposed and non-exposed neuroblastoma cells are shown in Figure 2A. Most cells from control cultures were colorless non-reactive while, ROFA exposed cells exhibited dark blue granules as a result of formazan precipitation. A dose-dependent response was observed after exposure to ROFA where a large proportion of

cells (60-70%) exposed to 10 and 50 µg/ml ROFA particles and even the lowest studied concentration (5 µg/ml) significantly increased the percentage of reactive positive cells as compared to untreated controls (p<0.001) (Figure 2B).

Sv pre-treatment reduced O_2^- generation being its effect more evident for ROFA 10 and 50 µg/ml (Figure 2B). Sv alone induced a slight but not significant increase in the levels of O_2^- compared to control values. Again as for cell viability, in all ROFA exposed cell cultures, Sv+Mv co-treatment showed no significant changes on this cellular parameter with respect to Sv alone (C = 13.92±1.59%; Sv+Mv = 12.18±0.62%; Sv+$ROFA_5$+Mv = 17.12±2.21%; Sv+$ROFA_{10}$+Mv = 29.40±0.51%; Sv+$ROFA_{50}$+Mv = 34.17±1.54%) .

Simvastatin enhances ROFA-induced antioxidant nuclear factor Nrf2 activation

The nuclear factor erythroid 2-related factor (Nrf2) is a transcription factor that controls the redox homeostatic gene regulatory network. In response to both endogenous and exogenous stresses caused by ROS, Nrf2 translocates to the cell nuclei enhancing the expression of a multitude of antioxidant and phase II enzymes that restore redox homeostasis.

To investigate the potential involvement of Nrf2, in the protective effect of Sv we evaluated Nrf2 nuclear translocation immunocytochemically. Figure 3A shows representative images of Nrf2 negative cell nuclei (Nrf2-) and Nrf2 positive cell nuclei (Nrf2+) indicative of Nrf2 nuclear translocation. SH-SY5Y cells exposed to ROFA showed an evident and significant dose-dependent Nrf2 nuclear translocation (Figure 3B).

It is noteworthy that Sv pretreatment *per se* modulates Nrf2 nuclear translocation since there was a marked increase in Nrf2+ cell nuclei immunostaining when compared non Sv pretreated control cells. Furthermore, the combination of Sv and 5 or 10 µg/ml ROFA dramatically increased the percentage of Nrf2+ cell nuclei as compared to ROFA alone (p< 0.001) (Figure 3B). On the contrary, cultured human neuroblastoma cells treated with a combination of Sv and the higher ROFA dose (50 µg/ml) showed a modest but still significant synergistic effect (p< 0.001) in comparison with non-Sv pretreated $ROFA_{50}$ cells.

Cell cultures co-treated with Sv+Mv showed no significant changes when compared to Sv alone (C = 13.81±1.15%; Sv+Mv = 46.86±3.44%; Sv+$ROFA_5$+Mv = 66.94±1.69%; Sv+$ROFA_{10}$+Mv = 85.02±4.83%; Sv+$ROFA_{50}$+Mv = 60.04±2.16%).

Simvastatin diminishes apoptotic cell death induced by ROFA

As described above, ROFA at concentrations ranging from 5 to 50 μg/ml after 24 hs induced a marked reduction in SH-SY5Y cell viability therefore, we sought to investigate cell death assessing apoptotic biomarkers (active caspase-3 and cleaved PARP) by a fluorescent immunocytochemical staining method.

Exposure to increasing concentrations of ROFA (5, 10, 50 μg/ml) induced a dose-dependent increment in apoptosis (Figure 4). Incubation with $ROFA_{50}$, the higher concentration used, resulted in 8.2 or 4.6-fold increase percentage of the positive cells for active caspase-3 ($p< 0.001$, Figure 4A) or cleaved PARP ($p< 0.001$, Figure 4B) over control values, respectively.

Sv pre-treatment was able to significantly diminish the percentage of apoptotic cells throughout the whole range of ROFA doses studied. As an example, the percentage of apoptotic cells evaluated either with active caspase-3 or cleaved PARP for Sv pretreated and exposed to $ROFA_{50}$, in comparison to non Sv pretreated cells, fell 2.4 or 1.8 fold respectively. Again, Sv+Mv co-treatment showed no significant changes on cell death immunocytochemically evaluated with respect to Sv alone (Caspase-3: C = 6.79±0.71%; Sv+Mv = 7.21±1.76%; Sv+$ROFA_5$+Mv = 11.24±1.28%; Sv+$ROFA_{10}$+Mv = 17.08±0.79%; Sv+$ROFA_{50}$+Mv = 22.79±1.68%; and PARP: C = 10.09±1.69%; Sv+Mv =7.69±1.02%; Sv+$ROFA_5$+Mv = 11.51±1.43%; Sv+$ROFA_{10}$+Mv = 20.06±1.35%; Sv+$ROFA_{50}$+Mv = 24.08±1.71%) .

Simvastatin reduces ROFA-induced senescence in SH-SY5Y cells

Senescence invokes a specific cell cycle profile that differs from most damage-induced arrest processes and is characterized by an enlarged cell size, expression of pH-

dependent beta-galactosidase activity and an altered pattern of gene expression. In our study, senescence was evaluated by senescence-associated β-galactosidase staining in control and exposed to ROFA (50 µg/ml) SH-SY5Y cells. Microphotographs show no reactive cells were observed in control cultures, while ROFA exposed cells showed reactivity as evidenced by the intense blue color (Figure 5A).

ROFA particles provoked a dose dependent increase in the percentage of senescent cells. On the contrary, pretreatment with Sv significantly reduced this cellular process in cultures exposed to 10 and 50 µg/ml (Figure 5B). SH-SY5Y cultures treated with both Sv+Mv showed no variations in comparison to Sv alone (C = 4.72±1.58%; Sv+Mv =8.16±2.16%; Sv+ROFA$_5$+Mv = 12.99±3.75%; Sv+ROFA$_{10}$+Mv = 20.36±1.42%; Sv+ROFA$_{50}$+Mv = 20.40±1.01%).

Discussion

Several reports have confirmed that the brain is a critical target of PM exposure implicating oxidative stress as a predisposing factor that links PM exposure and neurotoxic susceptibility (MohanKumar et al., 2008; Wilker et al., 2015). However, the underlying mechanisms and sequence of events that culminates in PM neurotoxicity remain poorly understood. In this context, we explored *in vitro*, the potential harmful effects of ROFA - a known ambient air particle pollutant - on SH-SY5Y, a human neuroblastoma cell line.

As it was shown by our group and others, ROFA is a PM rich in soluble transition metals and low concentration of organic compounds (Ferraro et al., 2011; Schroeder et al., 1987). Hence, ROFA is the most frequent combustion-derived particle used to evaluate the contribution of metallic traces on air pollution toxicity (Chen and Lippmann, 2009). In particular, it has been demonstrated that direct oxidant production from transition metals coated on ROFA surface may in turn, initiate oxidative imbalance (Carvalho et al., 2014; Ferraro et al., 2011).

In the presence of environmental stressors like PM, reactive oxygen species (ROS) play an important role in cell signaling of many physiological and pathophysiological

processes. The results obtained in this study indicate that *in vitro* exposure of SH-SY5Y cells to ROFA particles in doses ranging from 10 to 50 µg/ml significantly compromise cell viability and augments superoxide anion generation. Superoxide anion (O_2^-), one of the main ROS, can oxidize and hence damage lipids, proteins and DNA which in turn may lead to different cell death processes (Lushchak, V.I, 2014; Rahal, A. et al., 2014). Two specific forms of cell death have received increased attention regarding PM exposure (Kanduc et al., 2002), being apoptosis the first of these processes and necrosis the second one. Nonetheless, in response to different kinds of stress, mammal cells can also develop a proliferative arrest known as "cell senescence" (Chen et al., 2005). Senescent cells are able to induce neighboring cells towards this process through soluble factors known as SASP (senescence associated secretory phenotype). It has been documented that both apoptosis and senescence are interconnected and interconvertible cellular processes (Childs et al., 2014). Interestingly, in this study, we have shown that ROFA particles in neuroblastoma cell not only induced apoptosis (Figure 4) but also senescence (Figure 5).

Nrf2 regulates the basal and induced expression of an array of antioxidant response element (ARE) dependent genes to regulate the physiological outcomes of oxidant exposure (Montes et al., 2015). In our study, ROFA exposed SH-SY5Y cells showed a dose dependent Nrf2 activation, which could be a consequence of a redox imbalance probably caused by the O_2^- augmentation.

Chartoumpekis D et al., have shown that Sv lowers ROS by activating Nrf2 through the PI3K/Akt pathway (Chartoumpekis et al., 2010). In accordance, the rise of Nrf2 nuclear translocation in Sv pretreated SH-SY5Y cultures, suggests that in part, the protective effect observed may be mediated by an induction of the antioxidant defenses able to balance the production of O_2^- caused by ROFA exposure. Moreover, the rise in Nrf2 nuclear translocation was significantly higher in the Sv pretreated group, indicating that Sv *per se* was able to prime the cellular antioxidant defense system and counter particle - induced oxidative stress more efficiently. It is important to point out that, even though the immunostaining for Nrf2 is a useful method to detect the nuclear localization of Nrf2, the percentage of positive cells could

be misestimated. Therefore, another measurements of Nrf2 pathway activation in order to supplement and improve the results, as the assessment of nuclear Nrf2 protein levels by immunoblotting using nuclear protein extracts or the levels of Nrf2 target genes by western blot or real-time PCR (e.g. NQO1, GCLC etc) could be useful.

Based on ROFA neurotoxicity in human neuroblastoma cell culture, we found that pre-treatment with Sv provided a great protection. Our results suggest that for its efficacy in diminishing ROS production, Sv reduces apoptosis and senescence percentages thus, restoring viability levels.

It has to be pointed out that the preventive effect of Sv does not depend on the cholesterol synthesis pathway given that the addition of mevalonate did not modify the outcome for any of the parameters measured in this study.

In general, PM not only exerts imbalance of the oxidative metabolism but, inflammation as well. The onset of oxidative stress can cause altered cell signaling pathways which, may activate pro-inflammatory mediators (Donaldson et al., 2006). Particularly, associations between oxidative stress and inflammatory responses to particles have been widely described within the literature (Donaldson et al., 2005; Rahman and MacNee, 2000).

In brief, we found that ROFA on SH-SY5Y cells were able to increase not only the generation of O_2^-, but raise Nrf2 nuclear translocation, apoptosis and senescence in a dose dependent manner while reduces cell viability. On the contrary, in ROFA exposed neuroblastoma cell cultures upon treatment with Sv cell viability values were restored to basal level. In accordance, Sv pretreatment induced a reduction in positive staining percentage of apoptotic (Caspase-3 and PARP) as well as senescence biomarkers (SA- β-gal).

Therefore, we conclude that using an *in vitro* exposure system; pretreatment with simvastatin may provide neuroprotection against Residual Oil Fly Ash (air particulate matter) induced neurotoxicity independently of its ability to inhibit cholesterol synthesis.

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Legend to the figures

Figure 1. Preventive effect of simvastatin (Sv) on ROFA exposed SH-SY5Y cell culture. SHSY-5Y cells not pretreated, pretreated with Sv or pretreated with both Sv and mevalonate (Mv) were exposed to increasing doses of ROFA and evaluated for cell viability with the MTT test. The table depicts results shown in the graph. * = p <0.001 vs Control; # = p < 0.001 vs corresponding non-pretreated group.

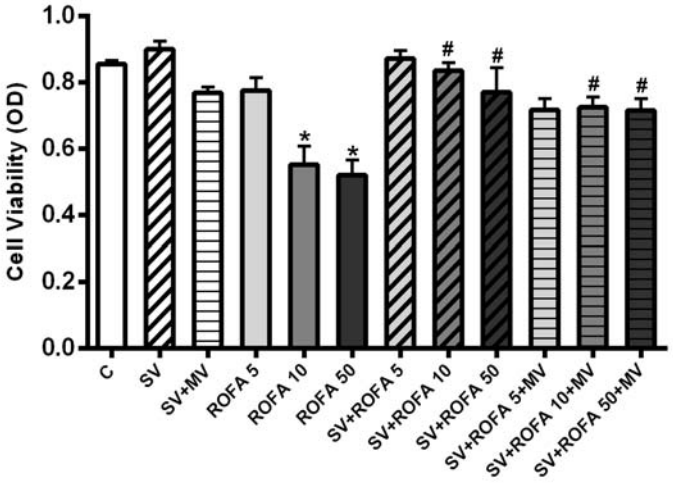
Figure 2. Effects of simvastatin (Sv) on ROFA induced oxidative stress. A) Microphotograph of reactive (left panel, $ROFA_{50}$) and non-reactive (right panel, Control) SH-SY5Y cells after NBT test (Magnification 1000x). B) SHSY-5Y cells not pretreated or pretreated with Sv were exposed to increasing doses of ROFA and evaluated for superoxide anion generation with the NBT test. * = p < 0.001 vs Control; # = p < 0.001 vs corresponding non-pretreated group.

Figure 3. Effects of simvastatin (Sv) on SH-SY5Y cells' antioxidant defense system induction. A) Microphotograph of Nrf2 immunocytochemical detection on control SH-SY5Y cells (negative, left panel) or Sv-pretreated cells (positive, right panel) (Magnification 1000x). B) SH-SY5Y cells not pretreated or pretreated with Sv were exposed to increasing doses of ROFA and evaluated immunocytochemically for Nrf2 induction. * = p < 0.001 vs Control; # = p < 0.001 vs corresponding non-pretreated group.

Figure 4. Effects of simvastatin (Sv) on ROFA-induced apoptosis. SH-SY5Y cells not pretreated or pretreated with Sv were exposed to increasing doses of ROFA and evaluated immunocytochemically for apoptosis. A) immunocytochemical detection of Caspase-3 active cleaved fragment. B) immunocytochemical detection of PARP cleaved fragment. * = p < 0.001 vs Control; # = p < 0.001 vs corresponding non-pretreated group.

Figure 5. Effects of simvastatin (Sv) on ROFA-induced cell senescence. A) Microphotograph of reactive (left panel, $ROFA_{50}$) and non-reactive (right panel, Control) SH-SY5Y cells after β-Gal activity test (Magnification 1000x). B) SH-SY5Y cells not pretreated or pretreated with Sv were exposed to increasing doses of ROFA and evaluated for cell senescence induction. * = p < 0.001 vs Control; # = p < 0.001 vs corresponding non-pretreated group.

Figure 1



Sample treatment	Mean ± SEM
C	0,855±0,011
Sv	0,899±0,024
Sv+Mv	0,769±0,017
ROFA 5	0,775±0,038
ROFA 10	0,552±0,055*
ROFA 50	0,520±0,045*
Sv+ROFA 5	0,872±0,024
Sv+ROFA 10	0,835±0,023#
Sv+ROFA 50	0,770±0,074#
Sv+Mv+ROFA 5	0,717±0,033
Sv+Mv+ROFA 10	0,724±0,031#
Sv+Mv+ROFA 50	0,715±0,036#

Figure 2

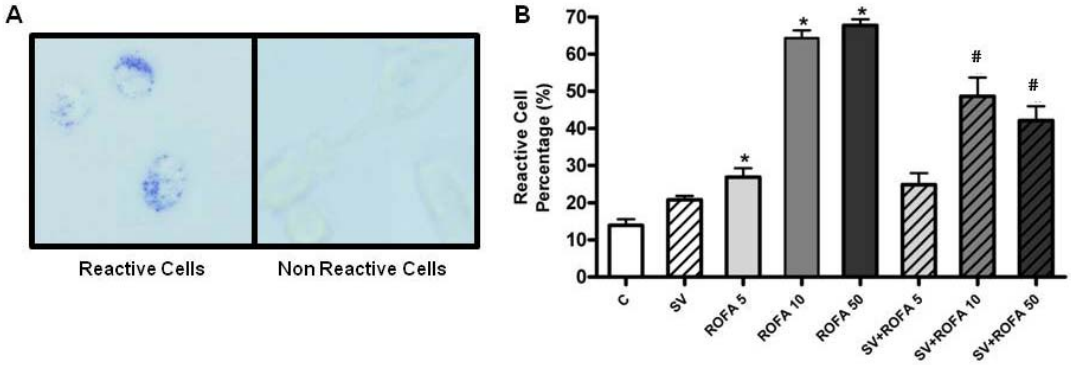


Figure 3

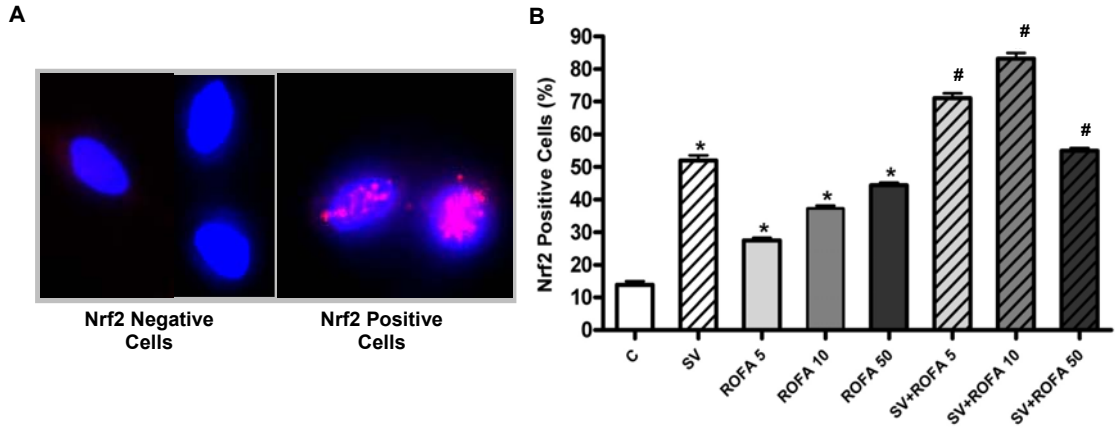


Figure 4

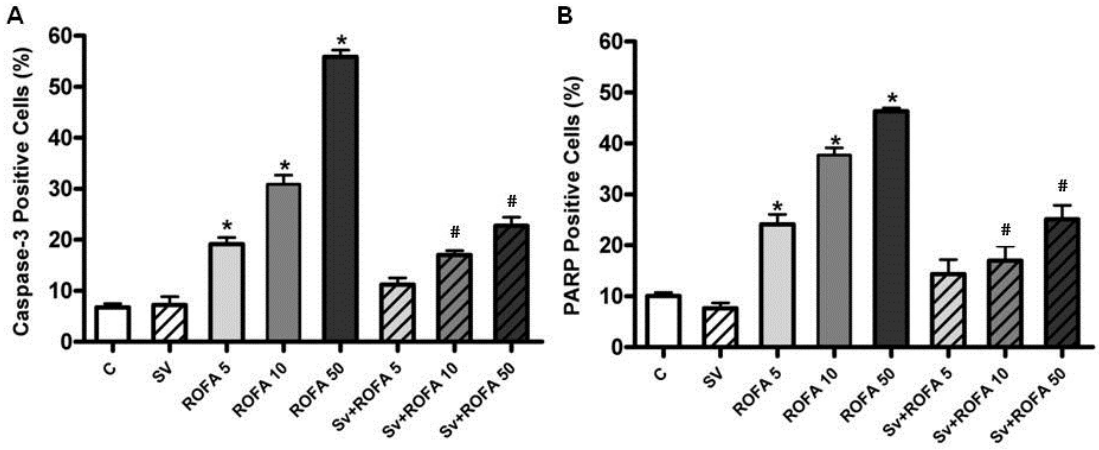


Figure 5