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Role of nicotine in oxidative stress

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ROLE OF NICOTINE IN OXIDATIVE STRESS

by

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ABSTRACT

Nicotine, a major alkaloid, is present in tobacco products as well as in smaller amounts in potatoes, tomatoes, and eggplants. Although it is the addictive compound in cigarettes, nicotine is believed to play a protective role in neurodegenerative disorders such as Parkinson's disease. Researchers have found that the short-term administration of nicotine may help improve the memory process by reducing oxidative stress in the brain. Our studies have shown that, by blocking Fenton's reaction, nicotine is able to inhibit free radical generation, so this research has focused on the antioxidant properties of nicotine. The cell lines of the alveoli, blood brain barrier, liver, and Chinese hamster ovaries were treated with 1mM of nicotine for 2 hours, and then the reactive oxygen species were measured. Results showed that the decrease in reactive oxygen species might be due to the inhibition of Fenton's reaction by metal chelation. The metal chelating property of nicotine was tested and its chelating ability was confirmed. Moreover, a study was made of the effects of nicotine on glutathione levels in alveolar cells that were treated with 0.4mM *tert*-butyl hydroperoxide for 3 hours. Results showed that nicotine prevented the depletion of glutathione levels in the tertiary butyl hydro peroxide treated group. These findings indicate that nicotine in both conventional and electronic cigarettes may alleviate symptoms of some neurodegenerative disorders by reducing oxidative stress caused by free radicals. However, the smoke from the burning tobacco in conventional cigarettes contains many harmful carcinogenic compounds in addition to nicotine. This is not the case with e-cigarettes, which also contain nicotine, but do not have the accompanying toxic materials.

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TABLE OF CONTENTS

Page

LIST OF ILLUSTRATIONS

Page

LIST OF ABBREVIATIONS

nAchR's	Nicotinic acetylcholine receptor's
GSH	Glutathione
GSSG	Glutathione- Oxidized form
ROS	Reactive oxygen species
DNA	Deoxyribo nucleic acid
HPLC	High performance liquid chromatography
HepG2	Human hepatocytes
CHO	Chinese hamster ovary cells
BBB	Blood brain barrier
DMEM	Dulbeco's modified eagle's medium
FBS	Fetal bovine serum
EDTA	Ethylene diamine tetra acetic acid
EBM	Endothelial basal medium
VEGF	Vascular endothelial growth factor
IGF	Insulin like growth factor
EGF	Epidermal growth factor
bFGF	Basic fibroblast growth factor
DCHF-DA	Dichloro dihydro fluorescein diacetate
t-BHP	tertiary butyl hydro peroxide
DETAPAC	Diethylene triamine penta acetic acid
SBB	Serine borate buffer
NPM	N-(1-pyrenyl)maleimide

MDA	Malondialdehyde
TCA	Trichloroacetic acid
BHT	Butylated hydroxy toluene
RPM	Rotations per minute
TBA	Thiobarbituric acid
PC12	Adrenal pheochromocytoma cells
e-cigarettes	Electronic cigarettes

1. INTRODUCTION

Nicotine, one of the most potent alkaloids found in significant amounts in tobacco leaves, is also present in lower levels in potatoes, tomatoes and eggplants. Chemically, nicotine, a tertiary amine, is composed of pyridine and pyrrolidine rings. It exists as two enantiomers in tobacco, levorotary *(S)* enantiomer and dextro *(R)*, with S being predominant (>99%).[1] (Figure 1.1). At a half-life of approximately 2 hours, it becomes extensively metabolized into cotinine (a major metabolite of nicotine).
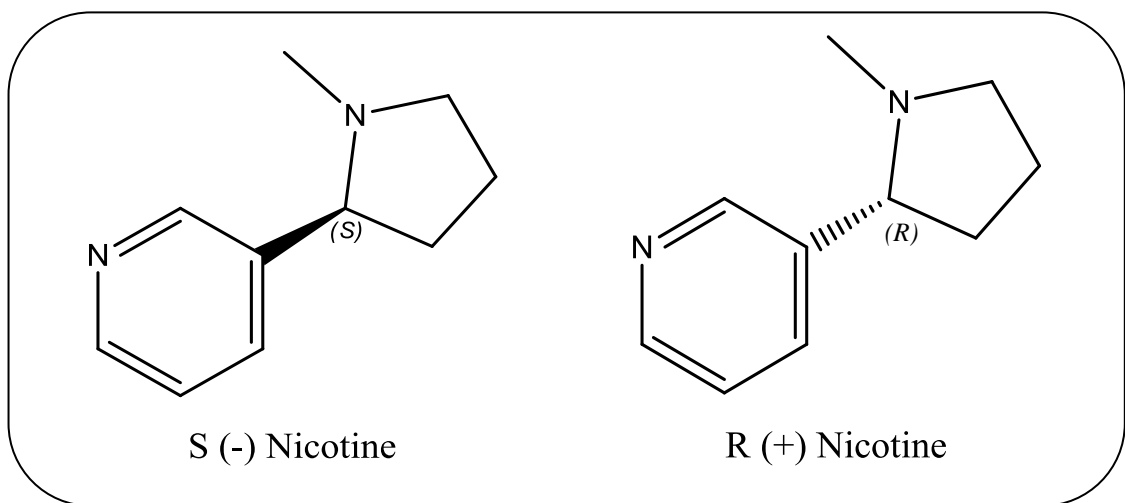
Figure 1.1 Enantiomers of nicotine.

1.1 PHARMACOLOGY OF NICOTINE

While nicotine, a para sympathomimetic alkaloid, can easily be absorbed by the lungs and readily pass through the blood brain barrier (BBB), its absorption through biological membranes is pH dependent.[2] Normally, nicotine is a weak base with a pKa of 8.0.[3] In an acidic environment, it becomes ionized and is not able to rapidly permeate biological membranes. Smoke from flue-cured tobacco (found in most conventional cigarettes) is acidic; hence it cannot permeate through the buccal membrane.[4] On the

other hand, smoke from air-cured tobaccos (found in cigars and some European cigarettes) is more alkaline (~pH 6.5), with a considerable amount of non-ionized nicotine that can readily permeate biological membranes. Researchers have found that the pH of cigarette smoke particulates is more alkaline, with a larger portion of the nicotine not being ionized, thereby facilitating rapid pulmonary absorption.[5] The nicotine from tobacco smoke is rapidly absorbed into the small airways and alveoli of the lungs, and then immediately enters and circulates in the blood.[6] The large surface area of the alveoli and the small airways in the lungs, along with the lung fluid (pH 7.4), facilitate rapid absorption of high amounts of nicotine by the membranes.

Nicotine, after entering pulmonary circulation, rapidly enters the brain[7] and then stereo-selectively binds to the nicotinic cholinergic receptors (nAChRs).[8] Stimulation of these receptors releases various neurotransmitters, including dopamine.[9] Dopamine, an important neurotransmitter that is responsible for the nicotine dependence and sensitivity mediated by the $\alpha_4\beta_2$ nAChRs. α_4 subunit, is an important determinant of sensitivity to nicotine.[10] On the other hand, the β_2 subunit mediates the release of dopamine and controls behavior.[11]

Studies have indicated that nicotine plays a potential role in the generation of reactive oxygen species, while some papers have shown its protective role in patients with brain disorders such as Parkinson's, Alzheimer's, and schizophrenia.[12] Parkinson's disease is the loss of neuromelanin containing dopamine from the substantia nigra pars compacta due to the further catalyzation by iron of the biochemical pathways of the neurotoxins.[13] Some reports demonstrated that the transdermal administration of nicotine

resulted in improvements in attention and information processing by Down's syndrome patients, when compared to healthy controls.[14] Researchers have also found that the chronic administration of nicotine might be beneficial to patients with dementia, where it acts as a cognitive enhancer.[15] These beneficial effects of nicotine might be due to its antioxidant mechanism that is primarily related to the presence of free iron.[16] Even though some research has indicated that cigarette smoking may have a potential advantage in treating neural disorders, like Parkinson's, it cannot achieve its full potential because of the dangerous toxicities associated with burning tobacco.[17]

With the advancement of technology, electronic cigarettes (e-cigarettes) have been introduced into the market and have become extremely popular among young people. In fact, sales of e-cigarette have increased from 50,000 in 2008 to 3.5 million in 2012.[18] They are considered to be a potential replacement for conventional cigarettes, which are associated with toxicities that are extremely carcinogenic. As nicotine from e-cigarettes enters the pulmonary circulation via the alveolar cells, the focus was on the role of nicotine in this cell line.

1.2   OXIDATIVE STRESS

Maintaining a balance between oxidants and antioxidants is a challenge for any living system in virulent environmental conditions, and a shift in balance could result in accumulation of reactive oxygen species (ROS) that further generates "oxidative stress." ROS result from many endogenous and exogenous sources, such as inflammatory cells, radiation, smoking, etc and they are believed to be the underlying cause for many disorders.[19]

1.2.1. Chemistry of ROS. Superoxide anion is considered as a primary ROS and it is generated as a consequence of either a metabolic process or by activation of oxygen by irradiation. These primary ROS will react with the other molecules to generate a secondary ROS via an enzyme or metal catalyzed process.[20] The superoxide anions generated undergo a dismutation reaction in the presence of a superoxide dismutase (SOD) enzyme, converting it into hydrogen peroxide and oxygen (Figure 1.2).

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

Figure 1.2 Dismutation reaction of a superoxide anion.

Redox active metals are known to closely link with the generation of free radicals.[21] The redox state of a cell is largely linked to an iron redox couple, and is maintained under strict physiological conditions.[22] However under excessive stressful conditions, an excess of superoxide releases free iron from enzymes, such as the dehydratase-lyase family which has [4Fe-4S] clusters[23]. Iron that is released participates in Fenton's reaction (Figure 1.3), promoting generation of hydroxyl radicals as a secondary ROS.[24, 25, 26, 27]

$$H_2O_2 \xrightarrow[\underset{Fe^{+2} \quad Fe^{+3}}{}]{} OH^{\cdot} + OH^-$$
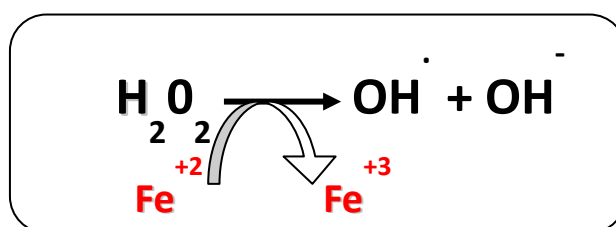
Figure 1.3 Fenton's reaction.

These generated hydroxyl radicals are highly reactive and have a half-life of less than 1 ns in aqueous solutions[28], which explains its highly reactivity towards anything in its vicinity. If hydroxyl radicals are generated close to DNA, they could react with DNA and result in DNA mutations, thereby causing cancer.[29] The hydroxyl radicals that are generated, not only react with DNA, but also with polyunsaturated fatty acid residues of phospholipids (main component of the cell membrane), which are extremely sensitive to oxidation[30, 31] and result in lipid peroxidation (Figure 1.4).
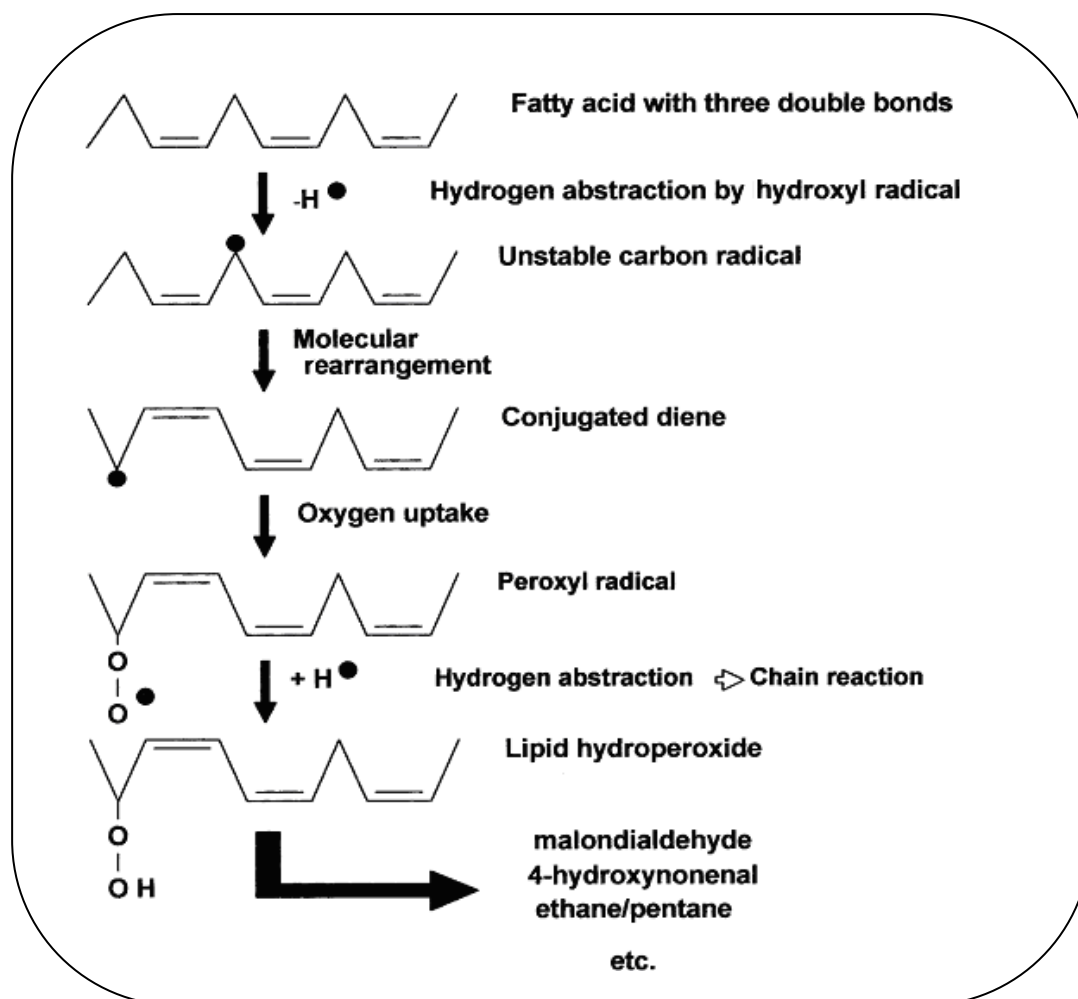
Figure 1.4 Mechanism of lipid peroxidation.[32]

1.2.2. Antioxidants and Their Role. In order to defend the body against the various ROS that are generated, a living system produces various antioxidants that can be either enzymatic or non-enzymatic. The modern way to define an antioxidant in a living system is "any substance that delays, prevents, or removes oxidative damage to a target molecule." [33]

Antioxidants can be broadly classified into

a. Enzymatic antioxidants:

- Superoxide dismutase (SOD)

- Catalase (CAT)

- Glutathione reductase (GR)

- Glutathione peroxidase (GPx)

The roles of these enzymatic antioxidants in oxidative stress are summarized in Figure 1.5.
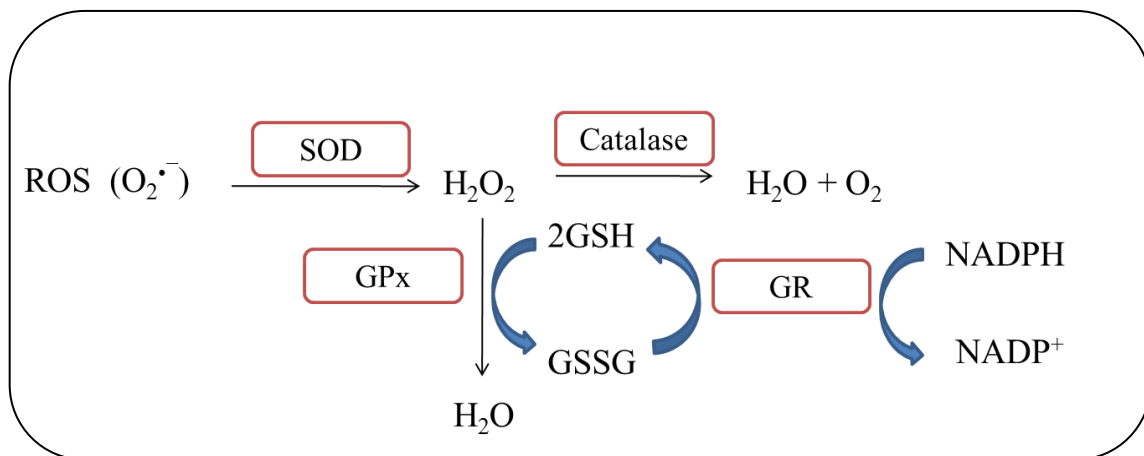
Figure 1.5 Enzymatic antioxidants working mechanisms.

b. Non-enzymatic antioxidants:

- • Thiol antioxidants

  - - endogenous thiols - glutathione (GSH), thioredoxin, lipoic acid, etc.

  - - exogenous thiols - N-acetyl cysteine (NAC), N-acetyl cysteine amide (NACA), etc.

- • Vitamins - E and C (ascorbic acid).

- • Metal chelators - Lactoferrin.

- • Other antioxidants - Carotenoids, Flavonoids, etc.

One of various non-enzymatic antioxidants, GSH is an important and major thiol antioxidant that is synthesized in the living system in order to defend against ROS. GSH, a tripeptide with a gamma peptide linkage between the cysteine amine group, is attached by normal peptide linkage to a glycine and the carboxyl group of the glutamate side-chain[34] (Figure 1.6). Most of the available GSH is kept in reduced form by glutathione reductase (GR) by the conversion of oxidized glutathione (GSSG). When there is oxidative stress, GSH is depleted, although it is constantly replenished by cells. In severe oxidative stress conditions, the rate of replenishment will not match the rate of depletion, resulting in excessive ROS pooling in cells and causing damage that may range from severe DNA damage to cell death.
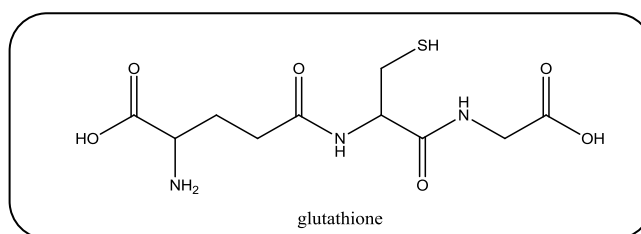
Figure 1.6 Structure of glutathione.

GSH antioxidant capability is due to the sulfur atom which can easily accommodate the loss of a single electron[35]. GSH antioxidant activity can be simplified as:

$$GSH + R^{\cdot} \longrightarrow GS^{\cdot} + RH$$
$$GS^{\cdot} + GS^{\cdot} \longrightarrow GSSG$$
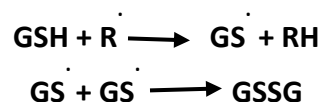
The protective roles of GSH in oxidative stress[36] are due to (i) cofactor (such as glutathione peroxidase and glutathione transferase) in detoxifying enzymes against oxidative stress, (ii) scavenge hydroxyl radical and singlet oxygen directly. Thus, GSH along with various enzymatic antioxidants maintains the balance between oxidants and pro-oxidants and protects cells from oxidative stress.

Many studies have revealed that cigarette smoking generates excessive free radicals that cause various diseases, including cancer.[37, 38, 39] Since the major ingredient in cigarettes is nicotine, a highly addictive component, smokers find it extremely difficult to quit. Even though a number of researchers are convinced as to the extent of the role of nicotine in oxidative stress, considerable research is still needed to determine its influence on our health and well-being.

Within tobacco smoke, (apart from nicotine), there are nearly 4,000 other compounds in various proportions. Studies have revealed that smoking tobacco generates numerous carcinogens, which are the major causes of various types of cancers, as well as promoting several oxidative stress disorders. When cigarettes are burned, Benzo[a]pyrene forms and enters the body. In the presence of the ubiquitous P-450 enzyme system, it is converted into (+) benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide, a known potent carcinogen (conversion shown in Figure 1.7).[40]
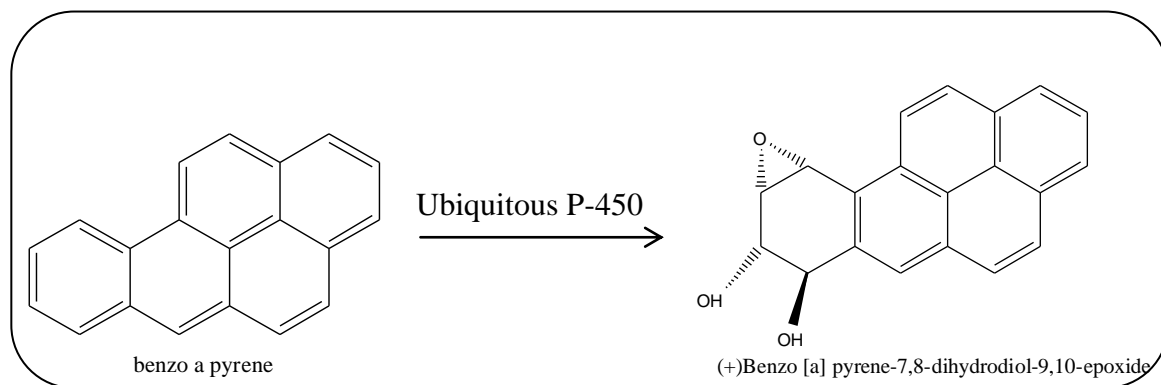
Figure 1.7 Conversion of benzo[a]pyrene.

As stated earlier the addictive properties of nicotine make quitting smoking a challenge for many people. With the introduction of e-cigarettes to the market, it is possible that smokers will benefit by using them instead of conventional cigarettes. The main addictive component, nicotine, is in both cigarettes, but the e-cigarettes do not have the toxic materials which promote carcinogens that accompany the nicotine in conventional cigarettes. Although various nicotine replacement therapies are marketed, such as nicotine patches and gum, these have not been successful because circulation of the nicotine from these replacements is slow and administration of sufficiently high levels of nicotine cannot be achieved. Because nicotine enters the body through alveolar cells, it is important to know the effects of nicotine on these cells, so a study was made of the role of nicotine in oxidative stress in these cells.

2. AIM OF STUDY

The primary aim of this study was to determine the role of nicotine in oxidative stress, particularly in the lung cells, where nicotine initially enters and circulates throughout the body.

And also an exploration was made of the potential role of nicotine as an antioxidant in the presence of free radical inducer tertiary butyl hydro peroxide.

3. EXPERIMENTS

3.1. CHEMICALS

High performance liquid chromatography (HPLC) grade solvents were obtained from Fisher Scientific, Waltham, MA. Soybean trypsin inhibitor was obtained from Life Technologies ^{TM}, New York, NY. All other chemicals were obtained from Sigma Aldrich, St. Louis, MO.

3.2. CELL CULTURE

3.2.1 Cell Lines.

3.2.1.1. Human liver hepatocellular carcinoma cells (HepG2). These cells were obtained from ATCC, Manassas, VA. They are adherent, epithelial-like cells that grow as monolayers and in small aggregates. They were derived from 15-year old male adolescent livers with differentiated hepatocellular carcinoma.

3.2.1.2. Chinese hamster ovary cells (CHO). These cells were obtained from ATCC, Manassas, VA. They are epithelial cells that grow as an adherent monolayer in a culture. This is the simplest and most popular cell line for carrying out screening studies.

3.2.1.3. Blood brain barrier cell line (BBB). The hCMEC/D3 cell line was used as a model for the human BBB. They were obtained as a gift from Prof. Pierre-Olivier Couraud. It was derived from human temporal lobe micro vessels that were isolated from tissue excised during a surgery to control of epilepsy.

3.2.1.4. Alveolar cell line (A549). These cells were purchased from ATCC, Manassas, VA. They are adherent, epithelial cells that grow as monolayers. They were derived from a 58-year old male Caucasian's lungs.

3.2.2. Culturing Cell Lines.

3.2.2.1. Culturing HepG2, CHO, and A549 cell lines. HepG2, CHO, and A549 cells were grown in a F-12: DMEM 1:1 nutrient media that was obtained from Sigma Aldrich, St.Louis, MO. A complete media was prepared with 10% fetal bovine serum (FBS), 2% pencilin, and 6.25ml of L-gultamine for a 500ml media. All cells from the liquid nitrogen were transferred to different $75cm^2$ corning flasks, which already contained 10ml of the complete media. These flasks were incubated with 5% CO_2 at 37° C. The media was changed every 48 hours until each flask reached 90% confluence. Once a flask was full, the cells were treated with 2ml of 1X Trypsin-EDTA and incubated for 3 minutes. Finally, trypsin action was blocked by using a soybean trypsin inhibitor.

3.2.2.2. Culturing BBB cells. BBB cells were obtained as a gift from Prof. Pierre-Olivier Couraud. They were grown in an EBM-2 basal media obtained from Lonza. A complete media was prepared by adding 5ml of penicillin- streptomycin, 12.5ml of FBS, 125µl of VEGF, 125µl of IGF, 125µl of EGF, 50µl of hydrocortisone, 5ml of 1M hepes buffer, and 5µl of bFGF to 500ml of basal media. The cells were cultured in the same manner as other cell lines were cultured.

3.3. EXPERIMENTAL PROCEDURES

3.3.1. Reactive Oxygen Species (ROS) Assay. ROS was measured in four different cell lines: BBB, CHO, HepG2 cells, and A549 cells. Cellular ROS were assayed in a Costar 96-well plate by using a Fluo-star Optima plate reader, in fluorescence mode, with an excitation wave length of 485 nm and an emission wave length of 520 nm, with 2', 7'-dichloro-dihydro-fluorescin diacetate (DCHF-DA) as a fluorescent dye (Figure 3.1). This could be enzymatically converted to the highly fluorescent compound, DCF, in the presence of ROS. Cells from the BBB, HepG2, CHO, and A549 cells were used to test the role of nicotine in generating ROS. In this test, the wells were seeded with 15,000 cells/well, with five replicates of each group. After 12 hours of seeding the cells, the media was replaced by DCFH-DA dye with a 40 μM concentration, prepared in a serum-free media, and then incubated for 1 hour. After 1 hour, a media with a dye was replaced by various concentrations of nicotine (1μM, 10μM, 100μM, 500μM, 1000μM) that had been prepared in a serum-free media. Cells were incubated with nicotine for 2 hours and the fluorescence was measured using a Fluo-star optima plate reader.

3.3.2. Cell Viability Assay. A cell viability assay was made of the A549 cell line by using a calcein AM dye, a non-fluorescent, hydrophobic compound, that easily permeates intact live cells. In the presence of esterases, it was converted to calcein, a hydrophilic, strongly fluorescent compound (Figure 3.2). Cell viability was carried out in a 96-well plate with 15,000 cells/well with five replicates. After seeding the cells in wells, the plates were incubated for 12 hours and then the cells were dosed with various concentrations of nicotine (2mM, 4mM, 5mM, 6mM) that had been prepared in a serum-free media and incubated for 24 hours. After 24 hours of incubation, the cells were

loaded with 2 µM of calcein AM dye, after removing the nicotine for 30 minutes. After 30 minutes of incubation, the plates were read using a Fluo-star Optima with 485 nm as the excitation wave length and 530 nm as the emission wave length. The fluorescent signal generated was proportional to the number of living cells.
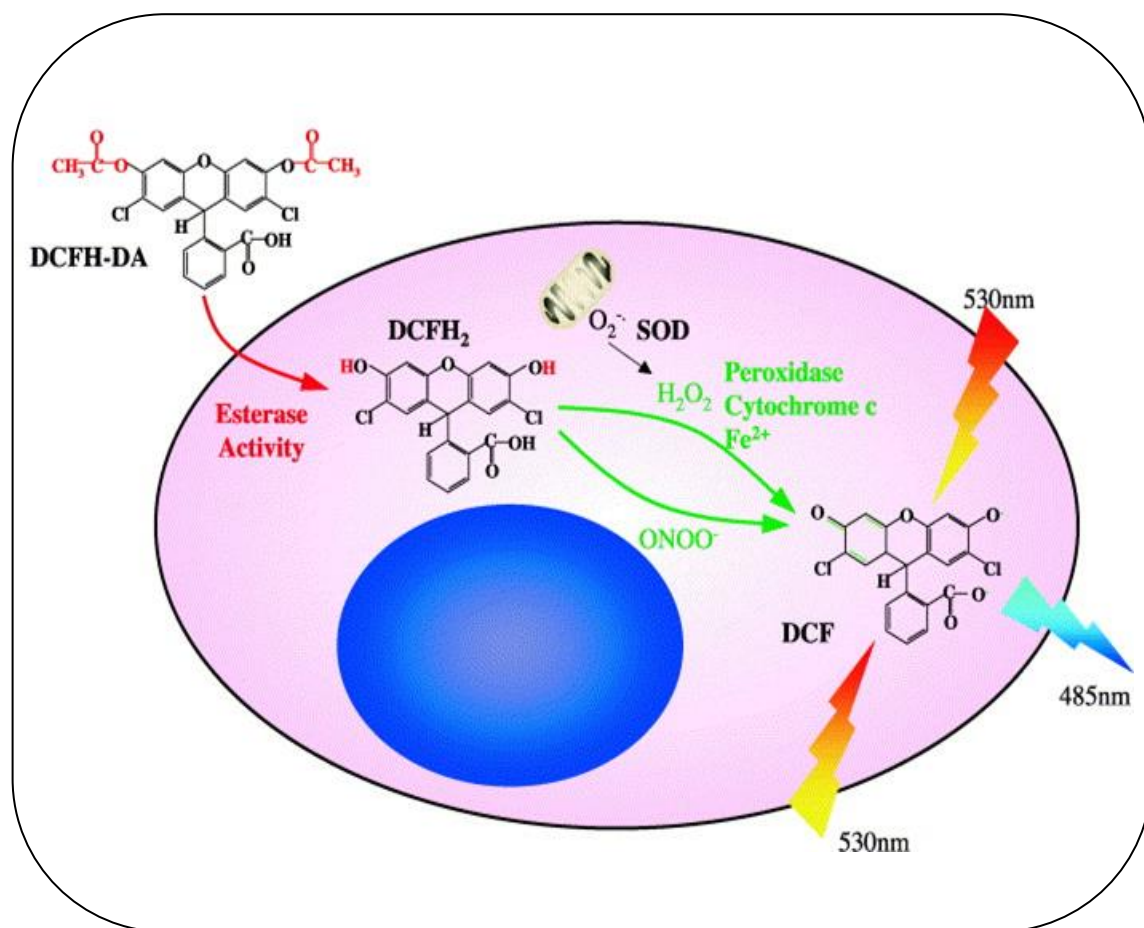
Figure 3.1 Measurement of ROS using DCFH-DA dye.[41]
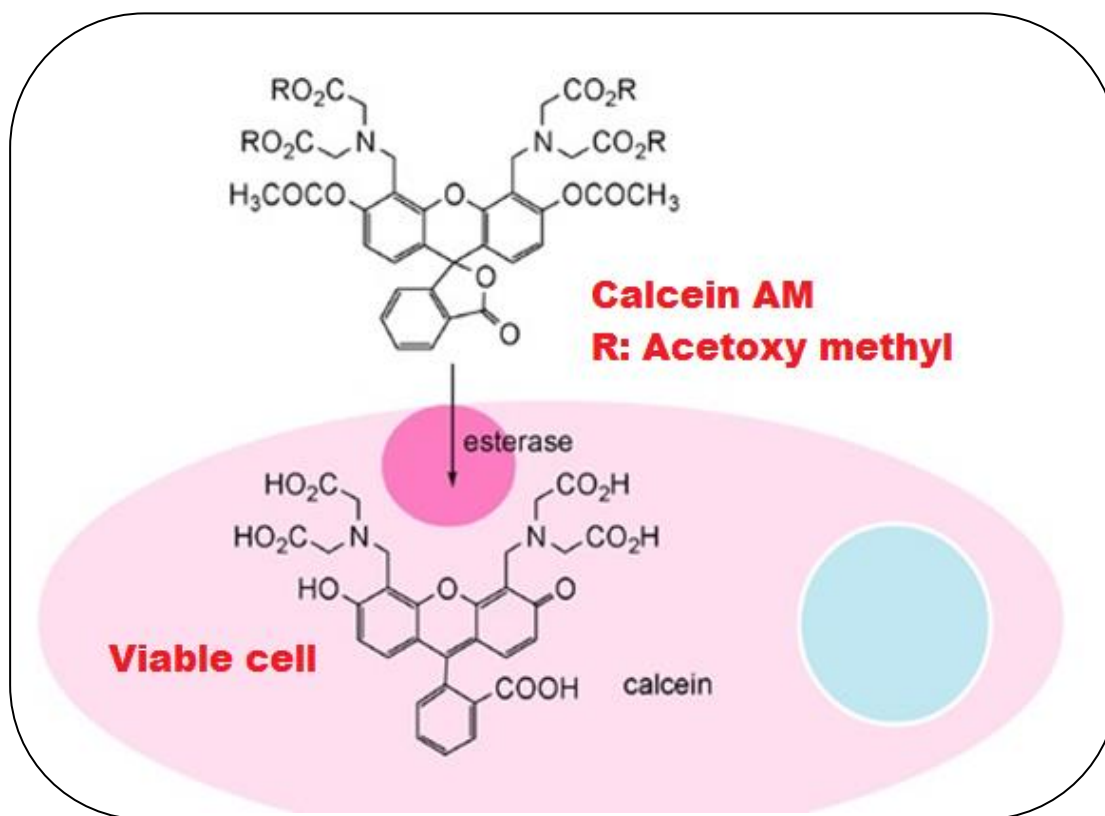
Figure 3.2 Cell Viability assay using Calcein AM dye.

3.3.3. Metal Chelating Capabilities. Iron chelating capabilities of samples were determined by the method of Dinis et al.[42] A solution containing 20µl of the sample and 0.38ml of water was added to 0.05ml of 2mM $FeCl_2$. The mixture was vortexed vigorously for 1 minute, and reaction was initiated by the addition of 0.2ml of 5mM of ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4"-disulphonic acid sodium salt] and 3.35ml of ethanol. The solution was incubated for 10 minutes and its color was monitored at 562 nm. The Fe^{+2} chelating activities of the samples were calculated as

$$\% \text{ Chelation} = [(A_o - A_1)/A_o] \times 100$$

where, A_o = Absorbance of the control, A_1 = Absorbance of the samples.

3.3.4. Glutathione (GSH) Measurement. Cellular levels of GSH were determined by using the method developed by RP-HPLC according to the method developed in our laboratory, Winters et al.[43] The HPLC system consisted of a FinniganTM Spectra System with a SCM 1000 vacuum membrane degasser, a FinniganTM Spectra System P2000 gradient pump, a FinniganTM Spectra System AS3000 autosampler, and a FinniganTM Spectra System FL3000 fluorescence detector (λ_{ex} = 330nm and λ_{em}=376nm) (Thermo Electron Corp., Austin, TX). The high performance liquid chromography (HPLC) column (Column Engineering, Ontario, CA) was 100 x 4.6 mm I.D and was packed with C18 packing material. Quantitation of the HPLC peaks was done using a Chromatopac Model CR 601 integrator (Shimadzu). The mobile phase was 30% water and 70% acetonitrile, and was adjusted to a pH 2 with acetic acid and O-phosphoric acid. The NPM (N-(1-pyrenyl)maleimide) derivatives of GSH were eluted from the column isocratically at a flow rate of 1ml/min. A549 cells were seeded in a 75 cm^2 flask and placed in an incubator to obtain 90% confluence, and then divided into four groups. Group A was control, Group B was nicotine, Group C was t-BHP, and group D was nicotine with t-BHP. Groups B and D were treated with nicotine in a serum-free media for 2 hours. After 2 hours of incubation, the Group B media was replaced with a serum-free media while Group C and Group D were treated with 0.4mM t-BHP for 3 hours and then incubated. Finally, cells are trypsinized and homogenate were prepared in a serine borate buffer (SBB) (100mM of Tris-HCl, 10mM of boric acid, 5mM of L-serine, 1mM of DETAPAC, pH 7.5). 50µl of this homogenate was added to 200µl of HPLC water and 750µl of NPM (1mM in acetonitrile). NPM (the derivatizing agent) reacted with free sulfhydryl groups to form fluorescent derivatives (Figure 3.3). After

incubation for 5 minutes, 2N HCl was added to the reaction mixture to stop the reaction. Derivatized samples were filtered through a 0.45µl acrodisc and then injected into a HPLC column. This adduct was detected by a fluorescent detector (Figure 3.4) that was connected to a HPLC column. Quantitation of the HPLC peaks was done using a Chromatopac Model CR 601 integrator.

N-(1-pyrenylmaleimdie)   N(-1-pyrenylmaleimide) thiol derivative
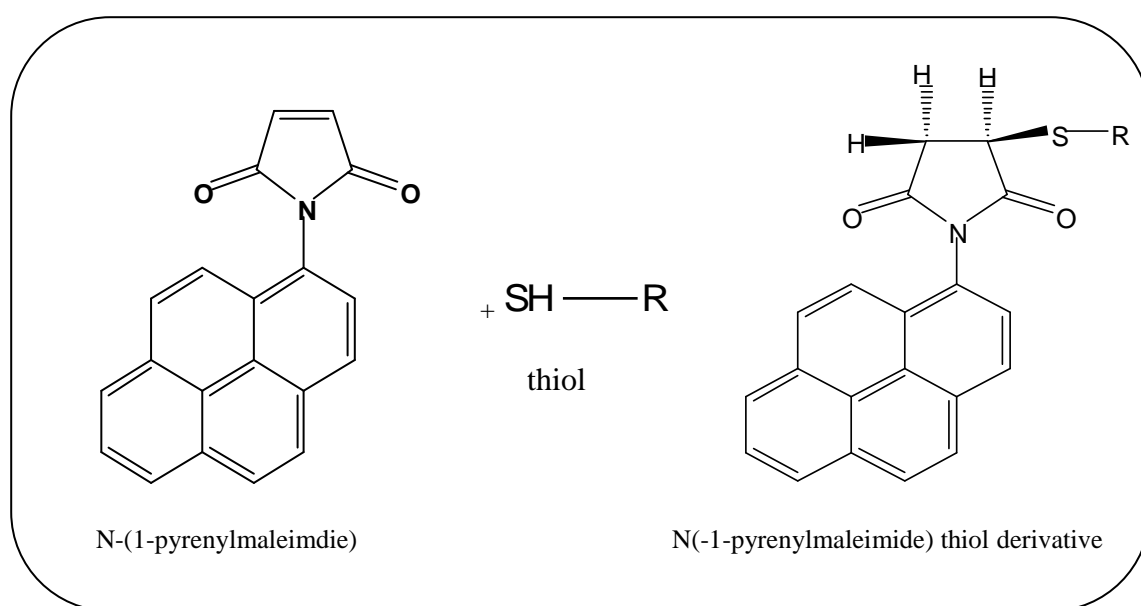
Figure 3.3 Derivatization of NPM.
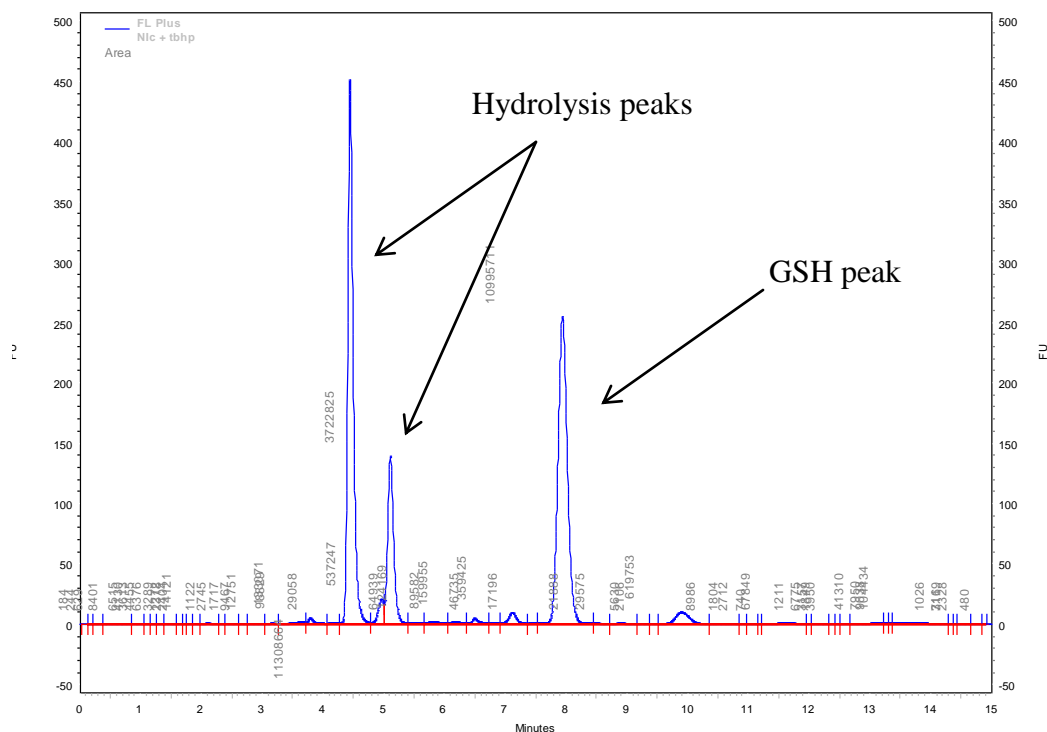
Figure 3.4 Chromatogram of A549 cells homogenate showing glutathione peak after derivatizing.

3.3.5. Protein Determination. The Bradford method was used to measure the protein levels of the samples using Coomassie Blue (Bio-Rad). The concentrated dye was diluted 1:5 (v/v) using SBB. 25µl of homogenate, that had been prepared in SBB, was mixed with 1ml of diluted dye and incubated for 5 minutes at room temperature. Various concentrations of a protein standard solution were prepared from a stock solution of bovine serum albumin (1mg/ml). To 25µl of stock solution, 1ml of diluted dye was added, and then the solution was incubated for 5 minutes. After incubation, the absorbance of the samples and the standard solution were measured at 595 nm. The unknown protein concentrations of the samples were calculated from a standard curve.

3.3.6. Malondialdehyde Assay. The amount of malondialdehyde (MDA) is one of the important parameters used to determine the degree of lipid peroxidation. 350 µl of cell homogenate were mixed with 100µl of 500ppm butylated hydroxy toluene (BHT), and then 550µl of 10% trichloroacetic acid (TCA) were mixed in pyrex tubes and boiled for 30 minutes. After the tubes were cooled in ice, they were centrifuged at 2,500 RPM for 5 minutes. 500µl of a saturated thiobarbituric acid (TBA) solution were mixed with 500µl of supernatant. After boiling again for 30 minutes (results in adducts, Figure 3.5), 500µl of this supernatant were placed in a test tube that contained 1.0ml of n-butanol. This mixture was vortexed for 5 minutes and then centrifuged for 5 minutes at 1,000 RPM. 200µl of the supernatant were placed in 96-well plates and then analyzed by using a fluorescent plate reader with an excitation wave length of 510nm and an emission wave length of 590nm.
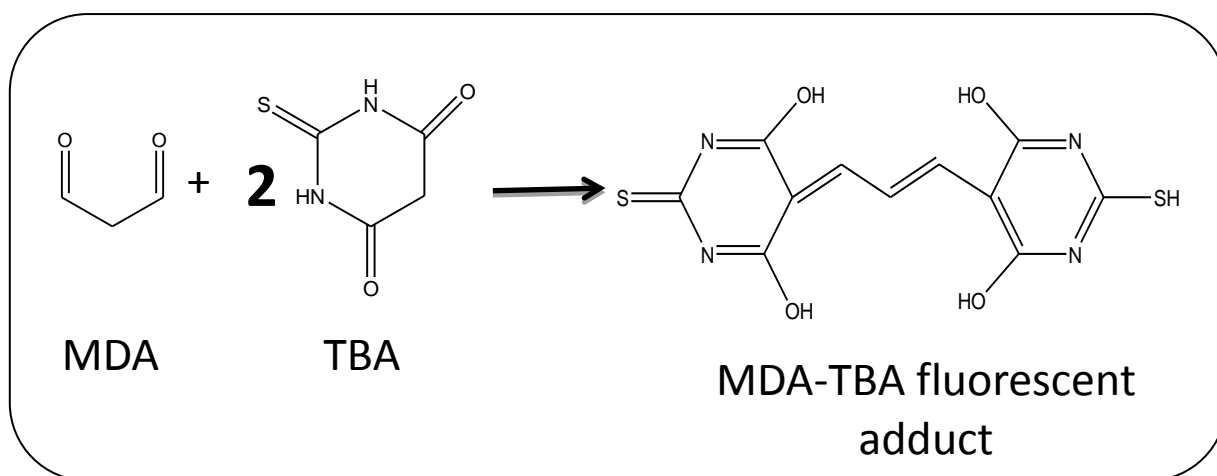

Figure 3.5 Formation of MDA-TBA fluorescent adducts.

3.3.7. Statistical Analysis. The data were given as the Mean ± SD. The one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were used to analyze the significance of the differences between the control and the experimental groups. Values of $p < 0.05$ were considered as significant.

4. RESULTS

4.1. GENERATION OF REACTIVE OXYGEN SPECIES (ROS) AND EFFECT OF NICOTINE

ROS were assayed in the cells of the BBB, HepG2, CHO, and A549. The cells were pretreated with DCFH-DA dye for 1 hour and then post treated with nicotine. Results indicated that all four cell lines did not cause any ROS when treated with different concentrations of nicotine (0.5μM, 1μM, 10μM, 100μM, 500μM). In fact, at higher concentrations, the nicotine significantly inhibited the generation of ROS in all cell lines, except in the CHO cells (Figures 4.1- 4.5).

4.2. CYTOTOXICITY OF NICOTINE

A cell viability assay was made of the alveolar cell line (A549). The cells were incubated with different concentrations of nicotine (2mM, 4mM, 5mM, and 6mM) for 24 hours. The results revealed that nicotine acted in a concentration-dependent manner on lung cells, but had a no significant toxic effect on the group treated with a 2mM nicotine concentration, as compared to the control group. Concentrations (beginning with 4mM) showed a significant toxic effect on these cells, as compared to the control group. Results are shown in Figure 4.6.

4.3. METAL CHELATION ASSAY

Nicotine was shown to have metal chelation properties when it was tested using ferrozine. With an increase in the concentration of nicotine, chelation increased proportionally, as shown in Figure 4.7.

4.4. GLUTATHIONE (GSH) ASSAY IN THE PRESENCE OF T-BHP AND THE EFFECT OF NICOTINE ON GLUTATHIONE IN T-BHP TREATED GROUP

GSH is an important parameter for determining the extent of oxidative stress in living systems. A549 cells were treated with nicotine and t-BHP. After incubation, the cells were trypsinized and then homogenized for assaying. Results indicated that nicotine treatment of the cells did not significantly deplete the GSH levels in the cells, as compared to the control. The group treated with t-BHP showed an increase in GSH levels when pretreated with nicotine, as indicated in the graphs in Figure 4.8.

4.5. MALONDIALDEHYDE ASSAY IN THE PRESENCE OF NICOTINE

MDA is an important parameter for determining the degree of damage to a cell. When excessive hydroxyl radicals are formed, they readily react with lipids which results in the formation of MDA as an end product. The MDA in A549 cells was measured fluorometrically by forming an adduct with TBA. The A549 cells that were treated with nicotine for 2 hours did not show any significant increase in MDA, as compared to the control, which indicates that nicotine alone may not induce lipid peroxidation in the A549 cell line as shown in Figure 4.9.

Figure 4.1 ROS generation and the effect of nicotine on A549 cells. The cells were treated with various concentrations of nicotine (0.1μM, 10μM, 100μM, 500μM, 1000μM) for 2 hours. 0.4mM t-BHP was used as a positive control and the ROS assay was carried out using DCF-DA fluorescence dye. Results are represented as mean ± SD (n=5). * Indicates significantly different from control group, P<0.05.
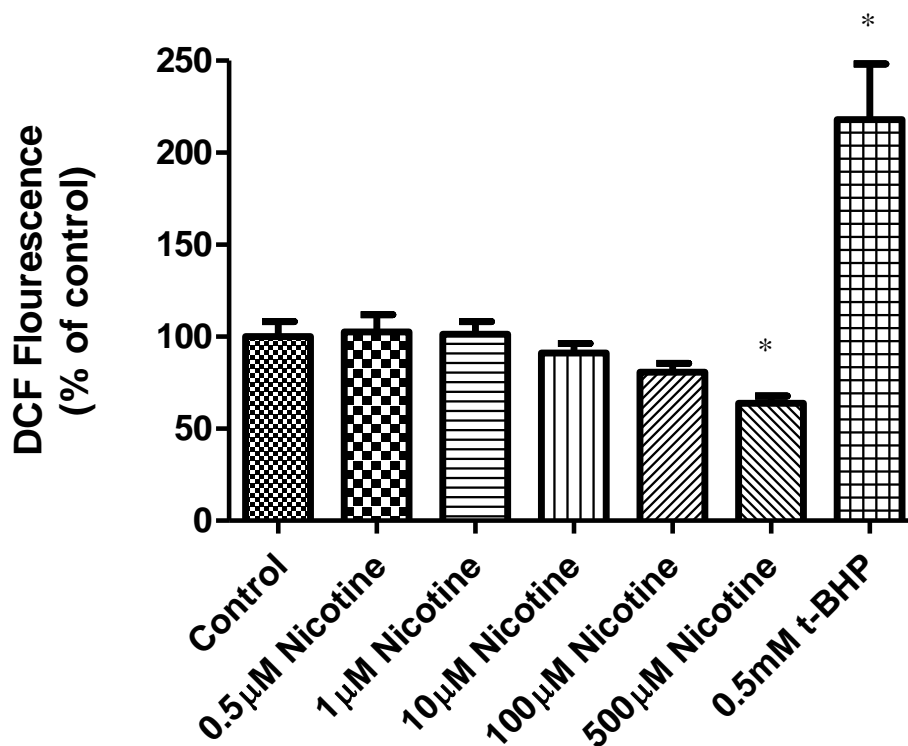
Figure 4.2 ROS generation and the effect of nicotine on BBB cells. The cells were treated with various concentrations of nicotine (0.1μM, 1μM, 10μM, 100μM, 500μM, 1000μM) for 2 hours. 0.5mM t-BHP was used as a positive control and the ROS assay was carried out using DCF-DA fluorescence dye. Results are represented as mean ± SD (n=5). * Indicates significantly different from control group, P<0.05.
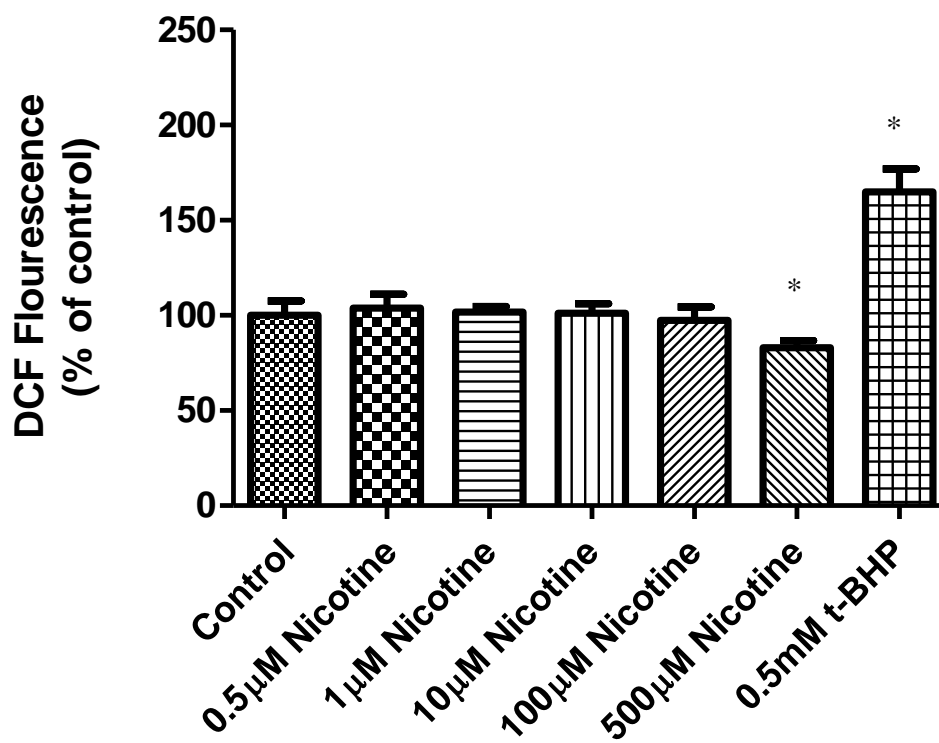
Figure 4.3 ROS generation and the effect of nicotine on HepG2 cells. The cells were treated with various concentrations of nicotine (0.1μM, 1μM, 10μM, 100μM, 500μM, 1000μM) for 2 hours. 0.5mM t-BHP was used as a positive control and the ROS assay was carried out using DCF-DA fluorescence dye. Results are represented as mean ± SD (n=5). * Indicates significantly different from control group, P<0.05.
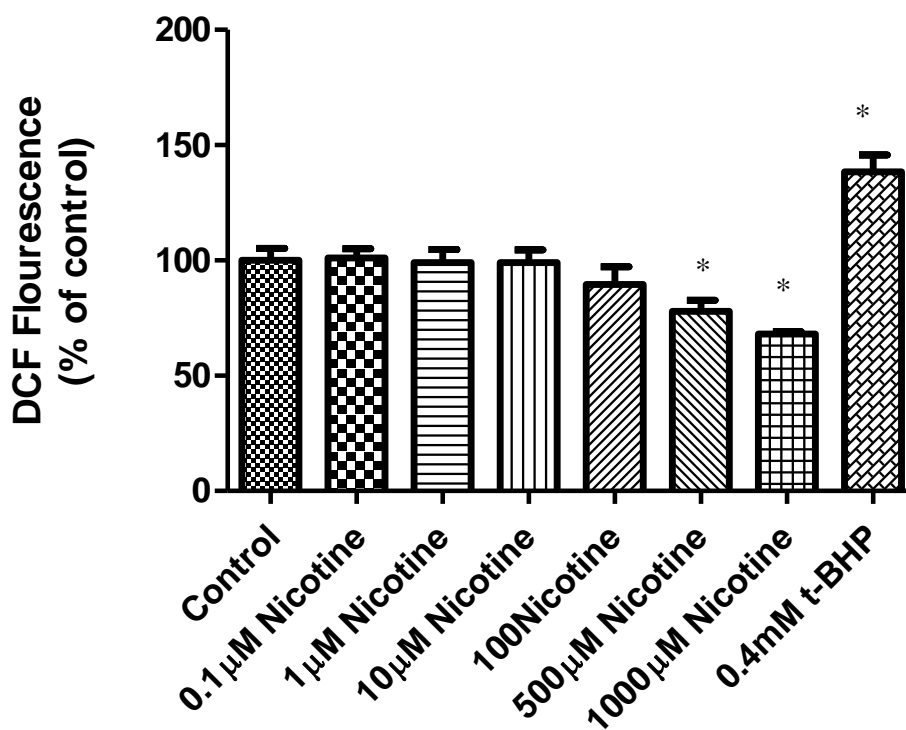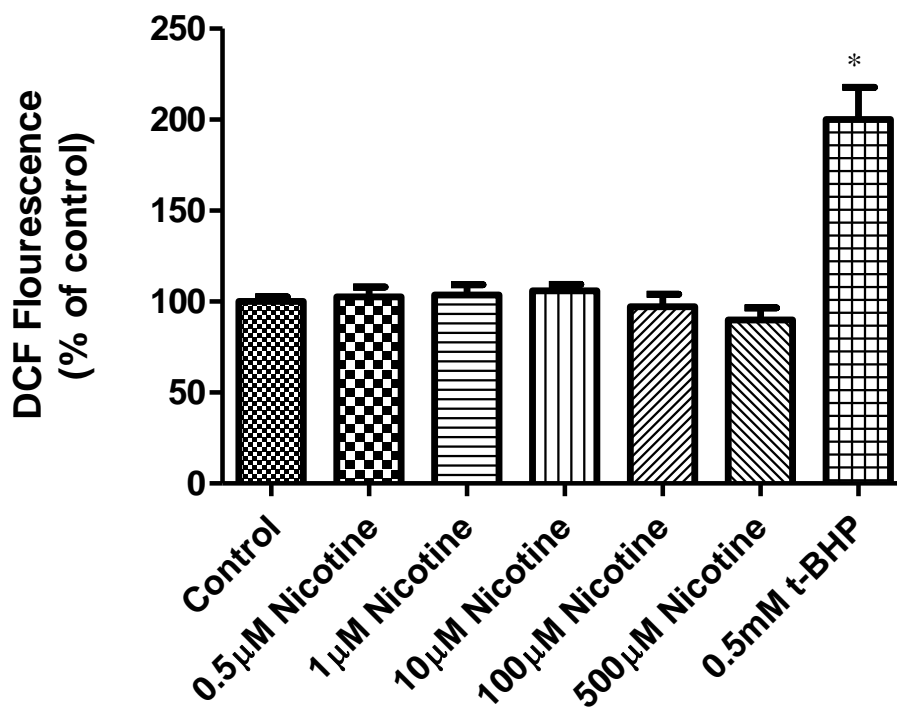
Figure 4.4 ROS generation and the effect of nicotine on CHO cells. The cells were treated with various concentrations of nicotine (0.1μM, 1μM, 10μM, 100μM, 500μM, 1000μM) for 2 hours. 0.5mM t-BHP was used as a positive control and the ROS assay was carried out using DCF-DA fluorescence dye. Results are represented as mean ± SD (n=5). * Indicates significantly different from control group, P<0.05.
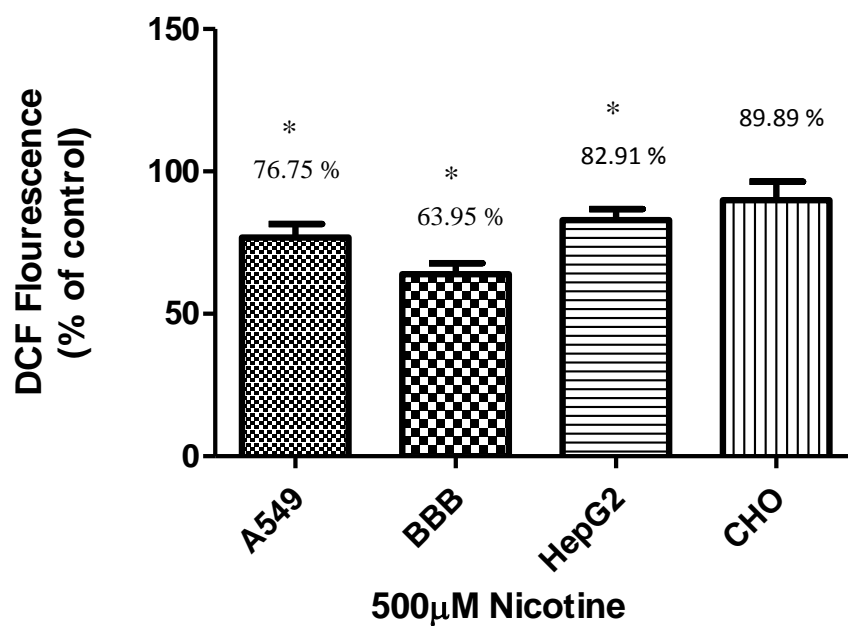
Figure 4.5 Summary of ROS assay of four different cell lines (A549, BBB, HepG2, CHO) with 500µM of nicotine. Graphs indicate percentage of fluorescence in different cell lines compared to that of control group. * Indicates significantly different from control group, P<0.05.
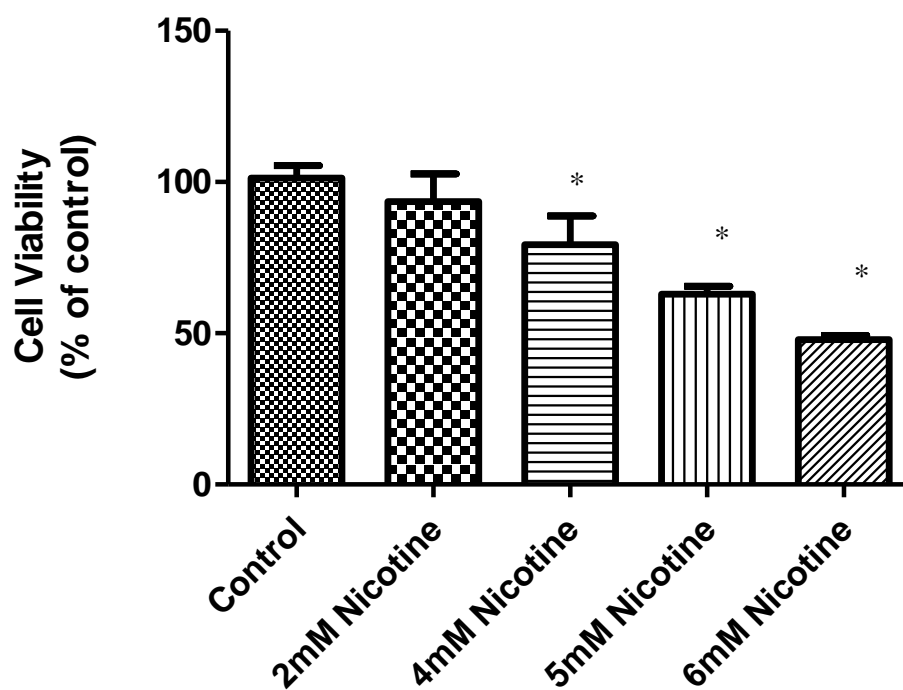
Figure 4.6 Cytotoxicity of nicotine on A549 cells. The cells were treated with various concentrations of nicotine (2mM, 4mM, 5mM, 6mM) for 24 hours. A cytotoxicity assay was carried out using calcein Am, fluorescence dye. Results are represented as mean ± SD (n=5). * Indicates significantly different from control group, $P<0.05$.
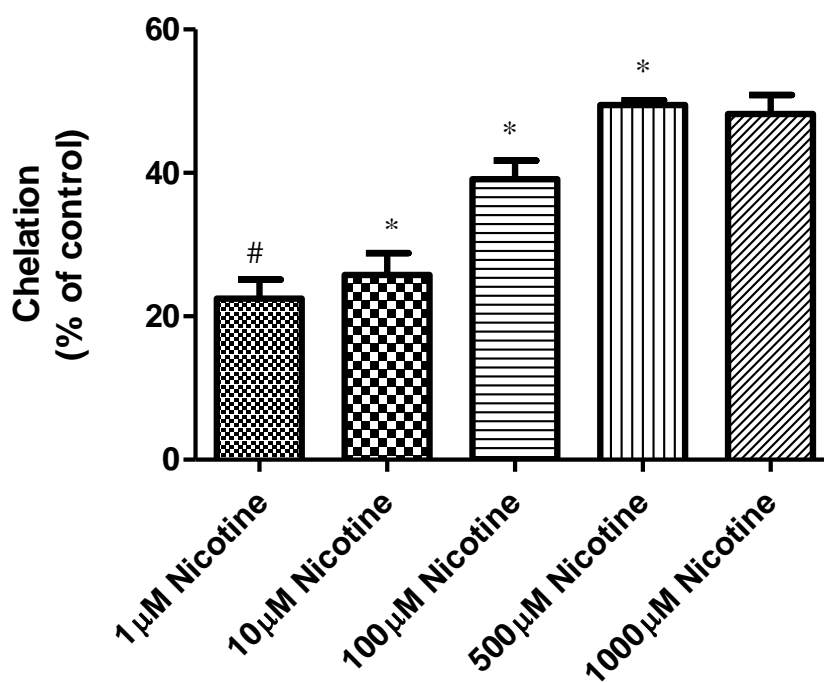
Figure 4.7 The metal chelating properties of nicotine. Experiment was carried out using various concentrations of nicotine (1μM, 10μM, 100μM, 500μM, 1000μM) with ferrozine. Results are represented as mean ± SD (n=4), * Indicates that groups were significantly different from each other. # indicates group significantly different from 100 μM, 500 μM and 1000μM nicotine (P<0.05).
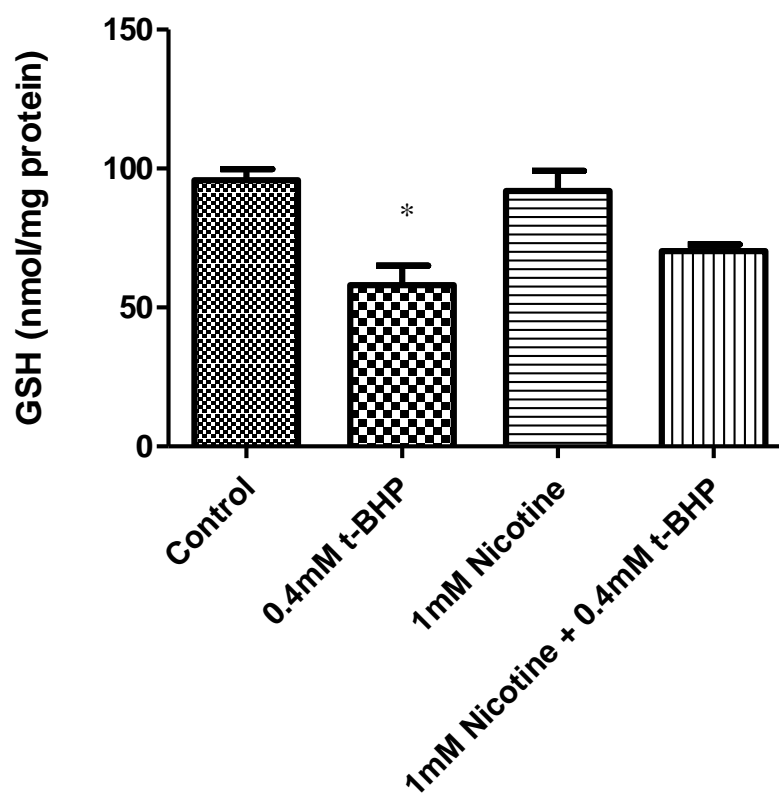
Figure 4.8 GSH levels after t-BHP treatment and the protective effect of nicotine. The cells were pretreated with 1mM nicotine (for 2 hours) and then treated with 0.4mM t-BHP for 3 hours and, finally the GSH was measured. Results are represented as mean ± SD. * Indicates groups are significantly different from control group (n=3), P<0.05.
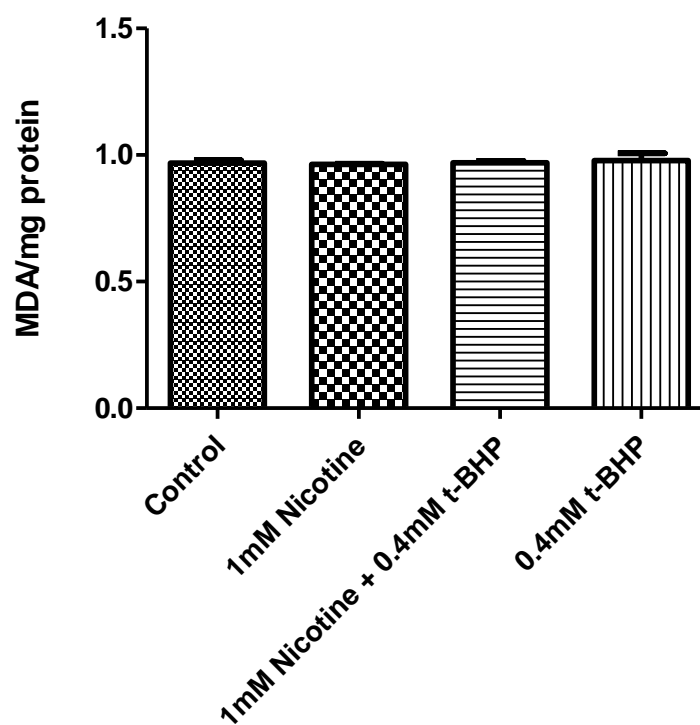
Figure 4.9 Malondialdehyde levels after t-BHP treatment and the effect of nicotine. The cells were pretreated with 1mM nicotine, treated with 0.4mM t-BHP for 3 hours, and then the MDA was measured. Results are represented as mean (n=3). No statistical significance was found between the groups.

5. DISCUSSION

Nicotine is a well-known parasympathomimetic alkaloidal drug that acts by releasing various neurotransmitters, such as acetylcholine, dopamine, vasopressin, etc. It is an addictive stimulant that is present in abundance in various tobacco products. Researchers have found that nicotine patch therapy relieves mild cognitive impairment in some individuals[44] while others have used animal models to show that nicotine may be protective in familial Parkinson's disease patients.[45] Considering the potential role of nicotine in treating various neurodegenerative disorders such as this, a study was made of the role of nicotine in oxidative stress by focusing on the alveolar cell line where the drug initially enters the body.

Nicotine was found by a few researchers to have a dual effect on oxidative stress in adrenal pheochromocytoma cells (PC12 cells)[45], while others identified the ability of nicotine to alleviate symptoms associated with Parkinson's disease.[46] This study was concentrated on the role of nicotine in alveolar, BBB, CHO, and HepG2 cells. The results indicated that nicotine did not produce any ROS, but that higher concentrations of nicotine (>500 μM) actually inhibited the generation of ROS, possibly by blocking Fenton's reaction by metal chelation. These findings are in agreement with studies done by Bridge MH[47]. All cell lines, except CHO cells, showed significant decreases in ROS production at concentrations of 500μM and above, as compared to the control group. CHO cells had less sensitivity toward nicotine even in the high concentrations and, hence, did not show a significant effect. This indicates that CHO cells may not have

nicotinic receptor. On the other hand, BBB cells showed greater sensitivity toward the 500μM nicotine concentration than any other cell line did.

Nicotine was found to be toxic when higher concentrations were used to treat cells. Toxicity was observed in a dose-dependent manner, when treatment was administered for 24 hours. Cells showed significantly decreased viability when treated with concentrations of more than 2mM of nicotine for 24 hours. Similar results were observed when leydig cells were treated with nicotine in various concentrations (from 1mM to 100 mM) for 24 hours.[48] The cells experienced death when they were treated with 5mM (or higher) concentrations of nicotine for 24 hours, with the death tentatively attributed to apoptosis when the caspase 3 pathway was activated. From this study, we concluded that nicotine does act in a dose-dependent manner and that it is also toxic to A549 cells when used in concentrations greater than 2mM.

Given the possibility of inhibiting Fenton's reaction by using nicotine, a study was made of the metal chelating effect of nicotine on iron. Results indicated that, with increase in the concentration of nicotine, there was a corresponding increase in metal chelation. This suggested that nicotine might be inhibiting Fenton's reaction by limiting the availability of iron, thereby preventing the generation of hydroxyl radicals. These properties of nicotine, and its ability to cross the BBB, explain its protective role for Parkinson's disease. In Parkinson's disease, dopamine becomes oxidized in the presence of the MAO-B enzyme, resulting in the generation of hydrogen peroxide as a byproduct.[5] In the presence of free iron, hydroxyl radicals were generated that resulted in cell damage (Figure 5.1).
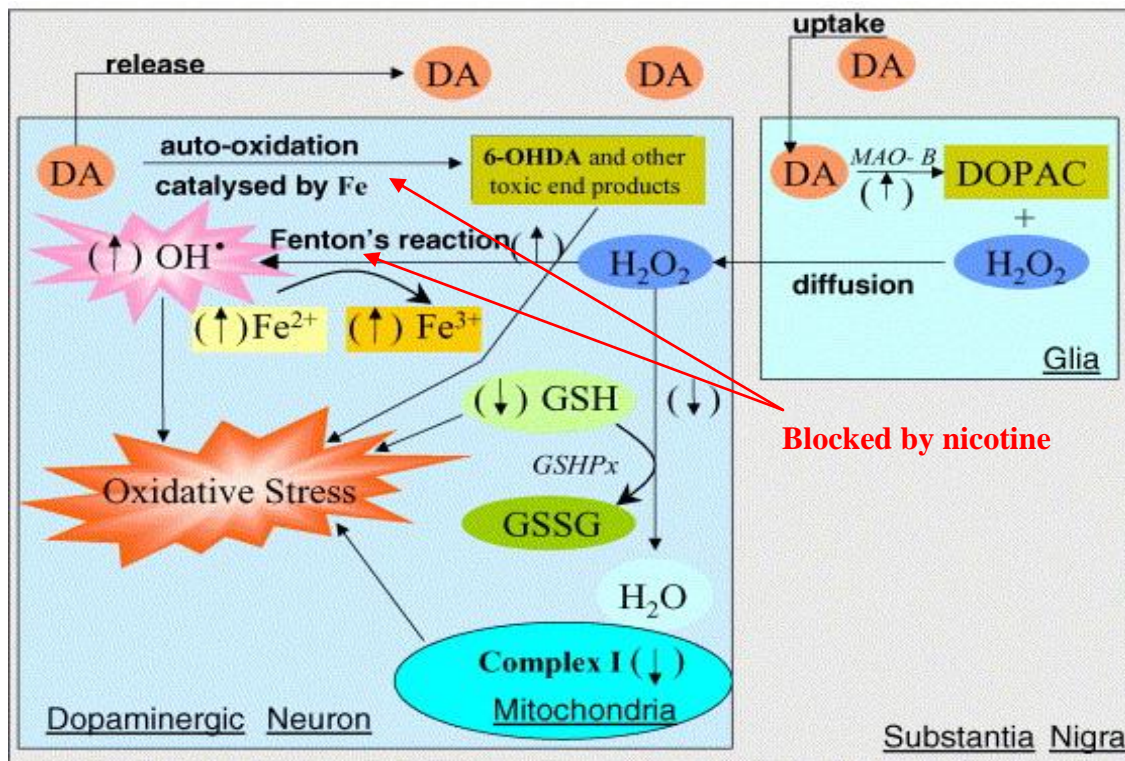
Figure 5.1 Role of Iron in progression of Parkinson's disease[49] and possible protective role of nicotine.

Since nicotine did not produce any ROS, the impact of nicotine on GSH levels was studied, and the potential of nicotine as an antioxidant was tested. The groups treated with nicotine alone did not show any significant decrease in GSH levels, when compared to that of the control group. However, the group pretreated with nicotine for 2 hours and, finally, with t-BHP for 3 hours, showed increased levels of GSH when compared to the group treated with t-BHP only. This result suggested that nicotine might have some protective role, in terms of oxidative stress, by preventing Fenton's reaction and, thereby, impeding the depletion of GSH levels in the cells.

Also, the role of nicotine in lipid peroxidation was reviewed. Results showed that nicotine did not cause any lipid peroxidation when treatment was for 2 hours, and there

was no significant difference when results were compared to those of the control group. This suggested that nicotine, when used as treatment for short periods of time, did not produce any hydroxyl radicals.

In summary, nicotine did not produce free radicals in A549 cells, BBB cells, HepG2 cells, and CHO cells; instead, it had a protective role by inhibiting generation of ROS. The GSH assay indicates that nicotine protects the cells from oxidative stress, possibly blocking Fenton's reaction by chelating the free iron and, thereby, preventing depletion of GSH.

6. CONCLUSION

These research findings suggest that nicotine does not generate ROS in alveolar, BBB, CHO and HepG2 cell lines, nor does it cause oxidative stress at the concentrations used (1μM, 10μM, 100μM, 500μM). In addition, nicotine does appear to prevent the depletion of GSH in the presence of a free radical inducer, thereby protecting cells from oxidative stress and, finally, death of the cells. The ability of nicotine to chelate iron (required for Fenton's reaction) may help explain how it inhibits free radical generation and alleviates symptoms (and possibly progression) of various neurodegenerative diseases. Because e-cigarettes contain nicotine, without the dangerous toxicities associated with burning tobacco, it is beneficial and may be a viable alternative to conventional cigarettes. Additional research is needed to completely understand the impact of nicotine and e-cigarettes on human health.

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