PROTECTIVE ROLE OF *N-TRANS*-FERULOYLTYRAMINE IN HYDROGEN PEROXIDE-INDUCED CELL DEATH

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ABSTRACT

Reactive oxygen species (ROS) are constantly produced by mitochondrial respiration under physiological conditions. Cells use the antioxidant enzyme system to scavenge ROS and prevent cellular damage and apoptosis. In many disease conditions such as Alzheimer's disease and Parkinson's disease, overproduction of ROS overwhelms cellular antioxidant capacity, and oxidative stress plays a pivotal role in the pathophysiology of those diseases. *N-trans*-feruloyltyramine (NTF), a plant-derived chemical, purified from a local medicinal tree called *Polyalthia suberosa*, possesses an antioxidant property that could decrease ROS. A recent study has concluded that NTF can effectively protect the auto-oxidation of linoleic acid. This study, therefore, aimed to evaluate the antioxidant effects of NTF in an *in vitro* cell model (SK-N-SH cells), induced with H_2O_2, and whether the reduction of the ROS level can lead to cytoprotection in our model. We investigated the protective effect of NTF by assessing cell viability using MTT assay, ROS level, and cell morphology. Apoptotic cell death was demonstrated by the level of the pro-apoptotic protein Bax, activated caspase-3, and the activity of caspase-3. Challenging SK-N-SH cells with 150µM of H_2O_2 increased the ROS level, induced cytotoxic changes, and decreased cell viability whereas preincubation with various concentrations of NTF in our model significantly reduced cellular ROS, attenuated cytotoxic cell death, level of Bax, and activated caspase-3. H_2O_2-induced increase in caspase-3 activity was also preventable by NTF pretreatment. According to these findings, NTF can significantly reduce cellular ROS and protect against H_2O_2 -mediated cytotoxicity and cell death in SK-N-SH cells.

KEY WORDS: *N-TRANS*-FERULOYLTYRAMINE/ANTIOXIDANT/APOPTOSIS/ REACTIVE OXYGEN SPECIES/ SK-N-SH CELLS

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LIST OF ABBREVIATIONS

AP-1	activator protein-1
Apaf-1	apoptotic protease-activating factor 1
ASK1	apoptosis signal-regulating kinase1
ANOVA	one-way analysis of variance
Bcl-2	B-cell leukemia/lymphoma-2 family members
BH3 only	Bcl-2 homology domain-3
CAT	catalses
CO_2	carbon dioxide
℃	degree celcius
BSA	bovine serum albumin
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
DISC	death-inducing signaling complex
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DCFH-DA	2, 7-dichlorodihydrofluorescein diacetate
DCF	2, 7-dichlorodihydrofluorescein
EDTA	Ethylenediaminetetraacetic acid
ECL	enhanced chemiluminescence
GPx	glutathione peroxidase
GSH	glutathione
H_2O_2	hydrogen peroxide
H_2O	water
HNE	4-hydroxynonenal
HRP	horseradish peroxidase
JNK	c-Jun N-terminal kinases
MDA	malondialdehyde
MEM	Minimum Essential Media

LIST OF ABBREVIATIONS (cont.)

μM	micromolar
MPTP	mitochondrial permeability transition protein
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NADPH	nicotinamide adenine dinucleotide phosphate
NTF	*N-trans*-feruloyltyramine
O_2	Oxygen
$O_2^{\cdot -}$	superoxide anion
$^{\cdot}OH$	hydroxyl radicals
PCD	programmed cell death
%	percentage
PBS	Phosphate-buffered saline
PVDF	Polyvinylidene Fluoride
pNA	p-nitroanilline
ROS	reactive oxygen species
RNS	reactive nitrogen species RNS
SOD	superoxide dismutase
SDS	sodium dodecyl sulfate
SEM	standard error of mean
TNFα	Tumor necrosis factor alpha

CHAPTER I
INTRODUCTION

Reactive oxygen species (ROS) are constantly produced by mitochondrial respiration under physiological condition during oxidative phosphorylation in ATP synthesis and approximately 1-2% of O_2 consumed is converted to ROS (1). Some of ROS species i.e. superoxide anion ($O_2^{\cdot -}$) and hydroxyl radicals ($^{\cdot}OH$), are highly reactive molecules (2). Existing in large amount of these highly reactive molecules in the cell results in oxidative damages to protein, lipid and DNA and eventually leading to apoptosis (3). Intracellular ROS production is greatly increased by various endogenous enzymatic systems, exposure to noxious substances or pathological conditions in the cell (4, 5). Normally, cells use the antioxidant defense system to scavenge ROS and to prevent cellular damages. In pathological conditions, excessive production of ROS overwhelms cellular antioxidant capacity and alteration in cellular redox homeostasis leading to oxidative stress (6). Oxidative stress is the state of the body, in which an imbalance between biochemical processes causes generation of reactive oxygen species (ROS) and elimination processes of ROS, antioxidant defense mechanisms (7). Oxidative stress has been demonstrated as a key condition underlying a number of human diseases including neurological conditions; i.e. Alzheimer's disease, Parkinson's disease and diseases with deranged cellular metabolism; i.e. cancer and diabetes mellitus, thalassemia and rheumatoid arthritis (8). In those diseases, the endogenous antioxidant defense systems appear to be relatively ineffective in combating oxidative stress (9, 10) and lead to deleterious effects. Searching for compounds with antioxidant activity becomes one strategy that could neutralise ROS production in the cell, prevent apoptotic cell death from oxidative stress (11). To date, more than 8000 phenolic structures have been identified. The core structural component of phenolic compound is aromatic ring(s) covalently linked with one or more hydroxyl constituents. Typically, antioxidant activity of phenolic compounds relies on their phenolic hydroxyl groups and their chemical structure that

function as free radical terminators. One of the polyphenolic compounds, *N-trans*-feruloyltyramine (NTF), (12) has been shown its antioxidant property (13, 14) and the free radical scavenging activity.

The goal of this study is to assess whether antioxidative effect of NTF could protect cell death in H_2O_2 insulted SK-N-SH cells. The cell model will be challenged with H_2O_2; as a result, the level of intracellular ROS is increased. As a consequence, cells will be subjected to apoptosis and result in reduced cell viability (Figure 1.1). If NTF is proven effective, decreased intracellular ROS represses cellular apoptosis and increased cell survival should be observed in NTF pretreated SK-N-SH cells (Figure 1.2).

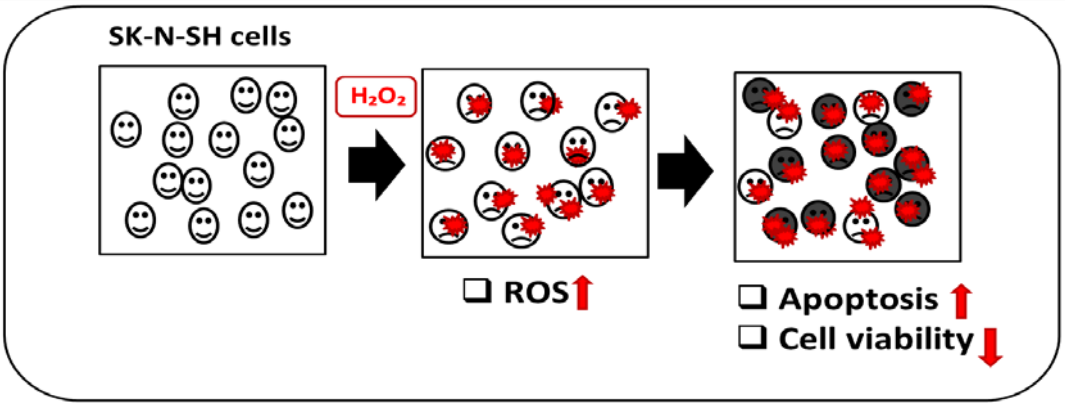


Figure 1.1 Proposed model showed effects of H_2O_2 induced toxicity in cultured SK-N-SH.

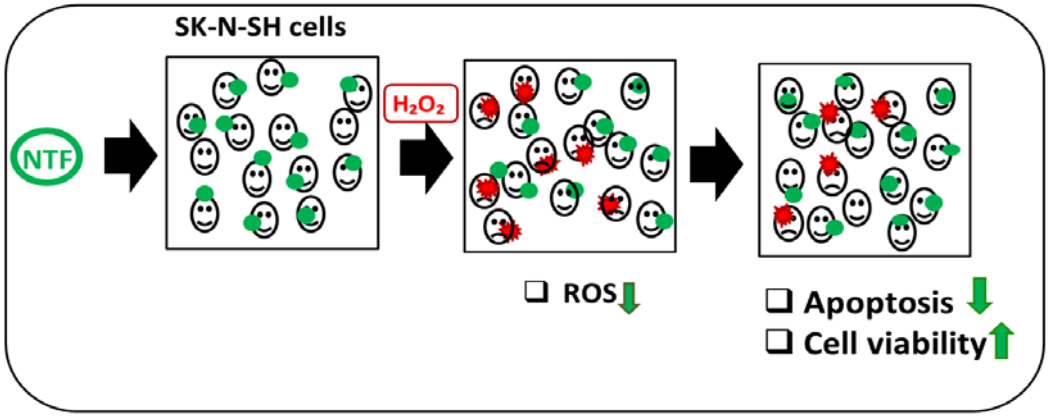


Figure 1.2 Proposed model showed protective role of NTF against H_2O_2 induced toxicity in cultured SK-N-SH.

CHAPTER II
OBJECTIVES

- To confirm an *in vitro* model of reactive oxygen species induced cell death.

- To examine the antioxidant effect of NTF in an optimised *in vitro* model.

- To study protective effect of NTF against H_2O_2 induced toxicity in an optimised *in vitro* cell model**.**

CHAPTER III
LITERATURE REVIEW

3.1 Free radicals and Reactive Oxygen Species

Free radicals are any molecular species whose one or more unpaired electrons exist independently in an orbital (15), as a result, free radicals are highly reactive molecules which can quickly react to other compounds rendering itself to be more stable, whereas the other compound loses its electron and consequently cause chain reaction of free radical and oxidative modifications of biomolecules (16). Free radicals are reactive oxygen species (ROS) or reactive nitrogen species (RNS). In focusing on ROS, they are group of electron stealing molecules that include oxygen in their chemistry, some of these species; i.e. $O_2^{\cdot -}$, $^{\cdot}OH$ or H_2O_2, are highly reactive molecules (2) (Figure 3.1), and the properties of these ROS molecules are shown in Table 3.1.

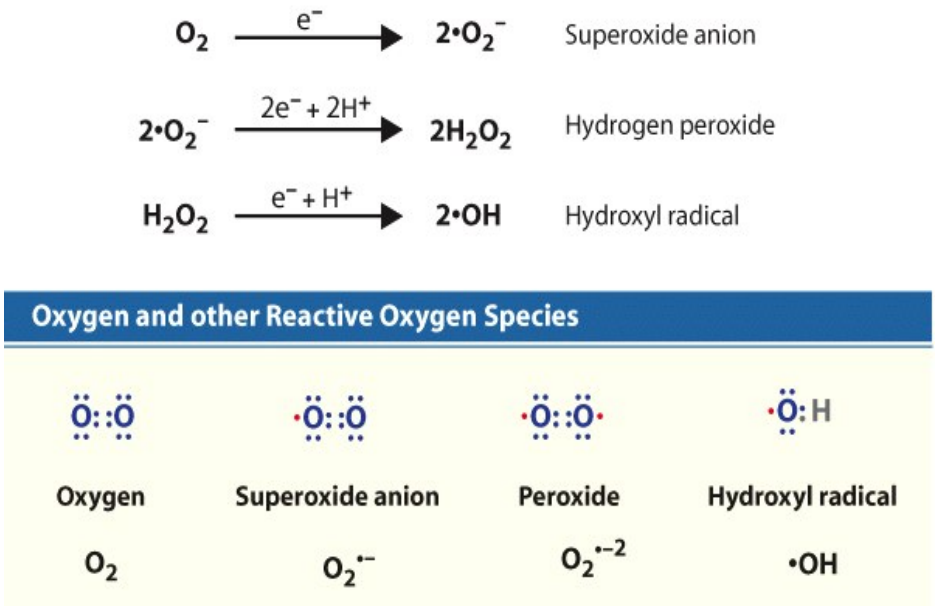


Figure 3.1 Structure of common Reactive Oxygen Species, taken form Halliwell B *et al*. (18).

Table 3.1 List of common reactive oxygen species and their properties, taken from Lobo V *et al* (17).

Reactive oxygen species	Formula	Properties
Superoxide anion	$O_2^{\cdot -}$	-One-electron reduction state of O_2 and produced from electron transport chain and formed in many autoxidation reactions. -Cannot diffuse far from the site of origin. -can generate other ROS.
Hydrogen peroxide	H_2O_2	-Two-electron reduction state and it is not a free radical but can generate free radicals by reacting with a transition metal (eg.,Fe^{2+}) -lipid soluble and can diffuse into and through cell membrane.
Hydroxyl radical	$^{\cdot}OH$	-Three-electron reduction state and extremely reactive species in attacking biologic molecules. -Formed from H_2O_2 via Fenton reaction in the present of metal ion.
Singlet oxygen	$^1O_2$	Oxygen with antiparallel spins. Produced at high oxygen tension from absorption of UV light.

3.2 Production of Cellular Reactive Oxygen Species

Reactive oxygen species are produced throughout human body as derivatives of cellular metabolisms (19, 20). Therefore, the mitochondrion is the major source of ROS production during oxidative phosphorylation in ATP synthesis (Figure 3.2). Superoxide anion ($O_2^{\cdot -}$), which is generated from mitochondrial electron transport chain, can also be produced by a number of cellular enzymes (15, 18); such as xanthine oxidase, lipoxygenase, cyclooxygenase, p-450 monooxygenase and NADH/NADPH oxidase (Figure 3.3). Generated superoxide anion is converted to hydrogen peroxide by superoxide dismutase. Subsequently, hydrogen peroxide (H_2O_2) can react with metal ions and generate extremely reactive hydroxyl radicals via Fenton

& Haber-Weiss reaction. Besides, cellular ROS generation can be trigger by a variety of external agents such as xenobiotic, ozone, toxin, irradiation, cigarette smoking, UV irradiation, drugs and air pollutants. The UV light can produce singlet oxygen by attacking to oxygen molecule (19). In cigarette smoke, lipophilic noxious active compounds such as, aldehydes and polycyclic aromatic hydrocarbons, transmigrate lipid bilayers into epithelial cells and augment rate of mitochondrial ROS production (20).

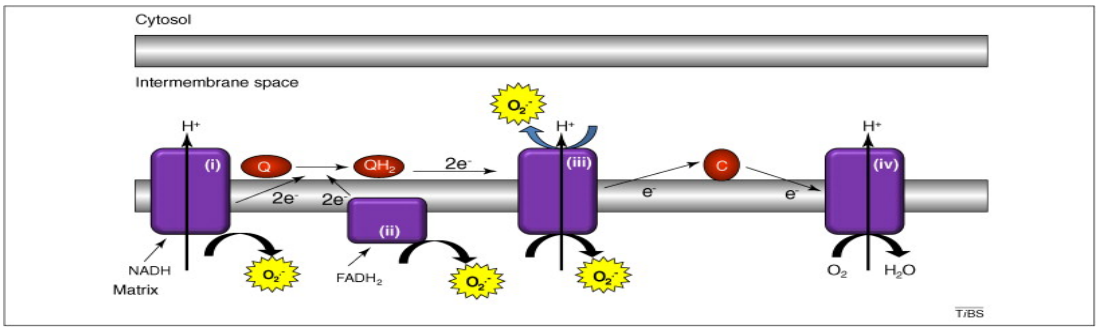


Figure 3.2 The major source of intracellular ROS production, taken from Turrens JF *et al* (18).

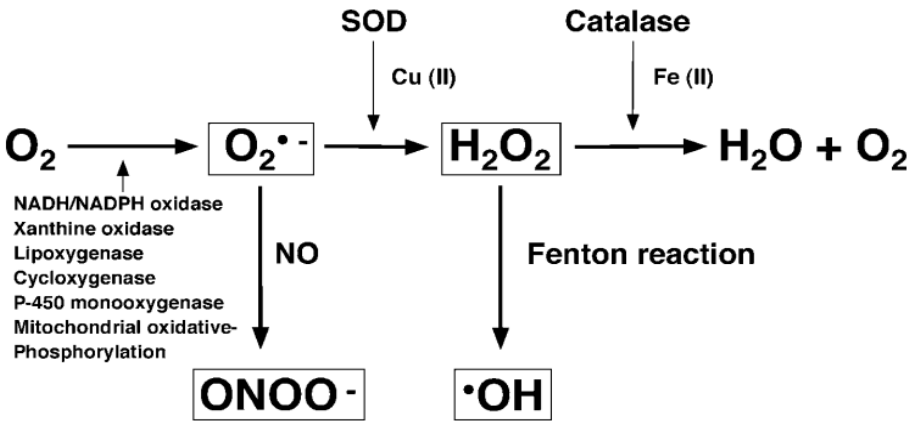


Figure 3.3 Cellular Reactive oxygen species production, taken from Kyaw M *et al* (21).

3.3 Cellular antioxidant systems

Mitochondria derived superoxide anion is catalysed by superoxide dismutase family, cellular antioxidant enzymes, in different compartment (Figure 3.4). SOD2 (MnSOD) in mitochondria matrix and SOD1 (Cu-ZnSOD) in intermembrane space converted superoxide anion ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2) respectively. The superoxide anion transported to the cytosol through the voltage-dependent mitochondrial anion channel (VDAC) can also be degraded by Cu-Zn-SOD. Hydrogen peroxide can undergo three pathways (22). Firstly, Fenton and Haber-Weiss reactions generate highly reactive species, hydroxyl radical (Figure 3.5). The other two pathways are mediated by antioxidant enzymes, i.e. catalase and glutathione peroxide. Catalase neutralises hydrogen peroxide into water and oxygen. Catalase is one of the most important enzymes in protecting the cell from ROS and possesses extremely rapid turnover rate (21). In the glutathione (GSH) system, hydrogen peroxide is reduced to water whereas glutathione (GSH) are transformed into oxidized GSH. These coupling reactions are catalyzed by glutathione peroxidase. Glutathione reductase is subsequently utilized to replenish reduced GSH (Figure 3.6).

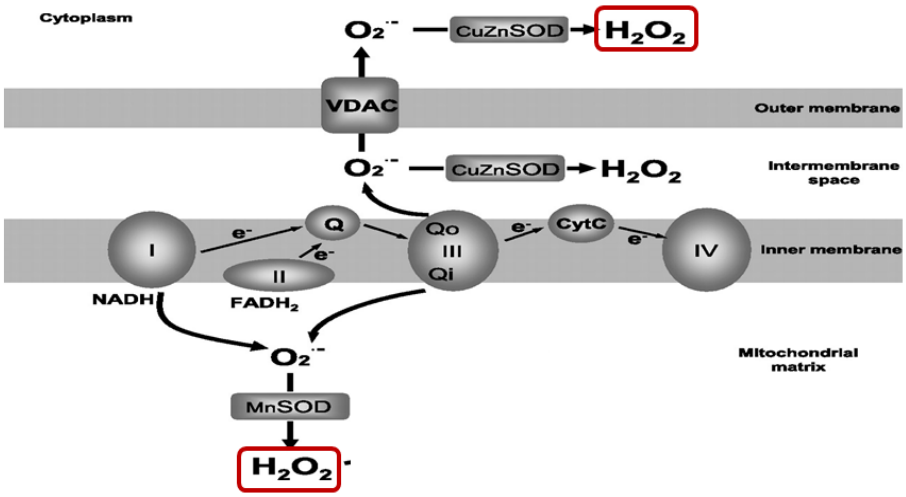


Figure 3.4 Mitochondria derived superoxide anion is dismutated to hydrogen peroxide by superoxide dismutase family, taken from Zhang DX *et al* (23).

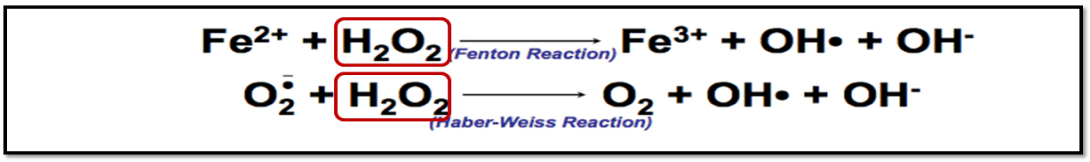


Figure 3.5 In the presence of metal ions, hydroxyl radical is generated through Fenton & Haber-Weiss reactions from hydrogen peroxide, taken from Kehrer JP et al (24).

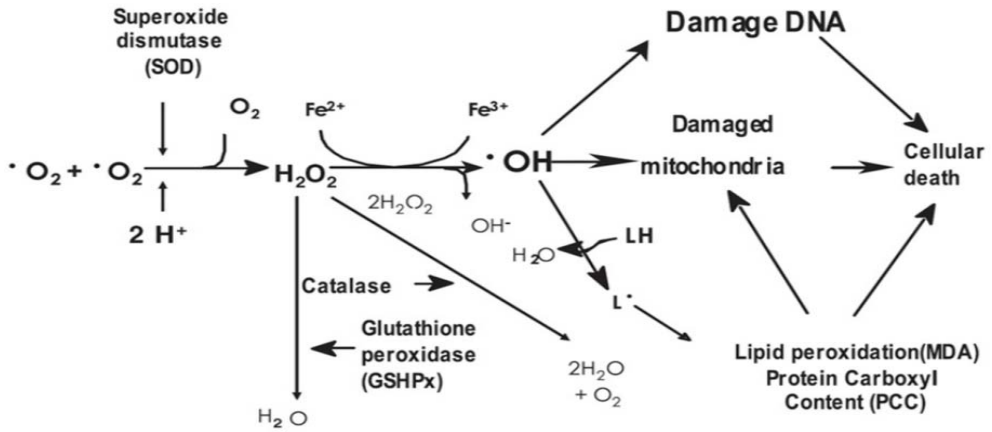


Figure 3.6 The schematic diagram of cellular ROS generation, elimination and oxidative damage, taken from Morón ÚM et al (25).

3.4 Oxidative stress

Oxidative stress can be defined as the state of the body in which excess of reactive oxygen species (ROS) is present. It occurs as an imbalance between generation and elimination of reactive oxygen species (26). That deleterious process, over production of ROS and the resulted oxidative stress can damage to cell structures, including lipids, proteins, and nucleic acids (27). In the process of oxidative damage to lipid, lipid peroxidation, free radicals steal electrons from the lipids especially polyunsaturated fatty acid in the cell membrane and then generate lipid radical. After that chain reaction of free radical continue and end up with the production of malondialdehyde (MDA) and 4-hydroxynonenal (HNE). The end products cause mutagenic (28). Extensive oxidative damage to nucleic acid can affect the deoxyribose

backbone, purine and pyrimidine bases lead to mutations (29). The most prominent DNA lesions resulting from reactive oxygen species is 8-OH-G formation (30). In the process of ROS induced oxidative damage to protein, sulfur containing amino acid such cysteine and methionine are mostly affected and consequently that can effect to protein structure and then functions of many proteins. (30, 31).

3.5 Apoptotic Cell Death

Apoptosis is defined as the process of programmed cell death (PCD) which can be recognized as morphological characteristic of the cells. Firstly, cell starts shrinkage and blebbing with chromatin condensation, but cell does not loss its integrity at this stage. After that, the cell becomes fragmented into formation of membrane bound vesicles (apoptosis body) that consists of cytosol, organelles and condensed chromatin. (Figure 3.7) Those morphological changes occur as the consequences of biochemical and molecular changes in an apoptotic cell such as mitochondrial cytochrome c release and activation of caspases.

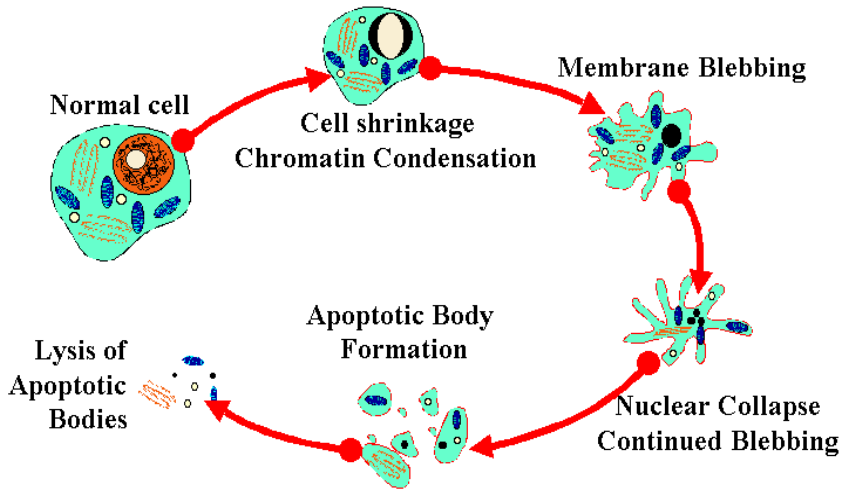


Figure 3.7 Morphological characteristics of apoptotic cell death, taken from Häcker G *et al* (32).

3.6 Control of cytochrome c release

Regulation of cytochrome c release from mitochondria and caspase activation are two key regulatory steps for apoptosis. Cytochrome c release is mainly controlled by B-cell leukemia/lymphoma-2 family members (33). There are three subfamilies; i.e. anti-apoptotic, pro-apoptotic and Bcl-2 homology domain-3 (BH3 only) group and their structural differences were shown in (Figure 3.8). Anti-apoptotic Bcl-2 subfamily contains four BH domains BH1, 2, 3 and 4 (34). The pro-apoptotic subfamily has two groups. The pro-apoptotic group Bax subfamily; i.e. Bax, Bak and Bok, contains BH1, BH2, BH3 and transmembrane domains.

Anti-apoptotic members such as Bcl-2 inhibit the pro-apoptotic proteins; i.e. Bax and Bak in the normal state. However, in cellular apoptosis, BH3-only members are stimulated by transcriptional up regulation of Bax subfamily members and halt the anti-apoptotic Bcl-2 members from antagonizing proapoptotic members. In addition, cytosolic monomer proteins, Bax subfamily members, change conformation due to apoptotic stimuli and subsequently oligomerization of Bax and Bak occurs and leads to the mitochondrial membrane pore formation. Eventually, increased cytosolic cytochrome c activate the caspase cascade through apoptosome complex (Figure 3.9) (35).

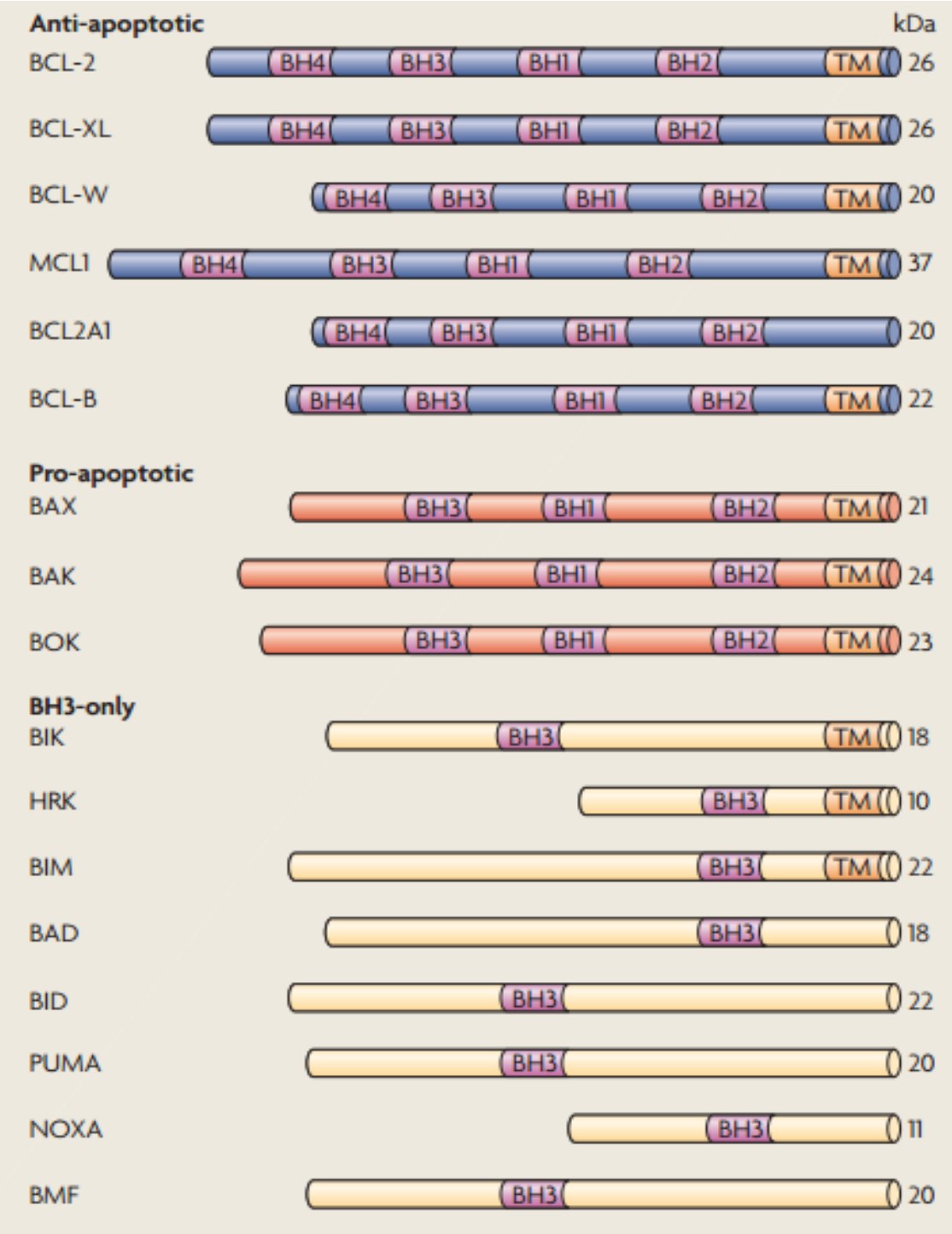


Figure 3.8 Regulators of cells survival and cells death: Bcl-2 family proteins, taken from Taylor RC *et al* (36).

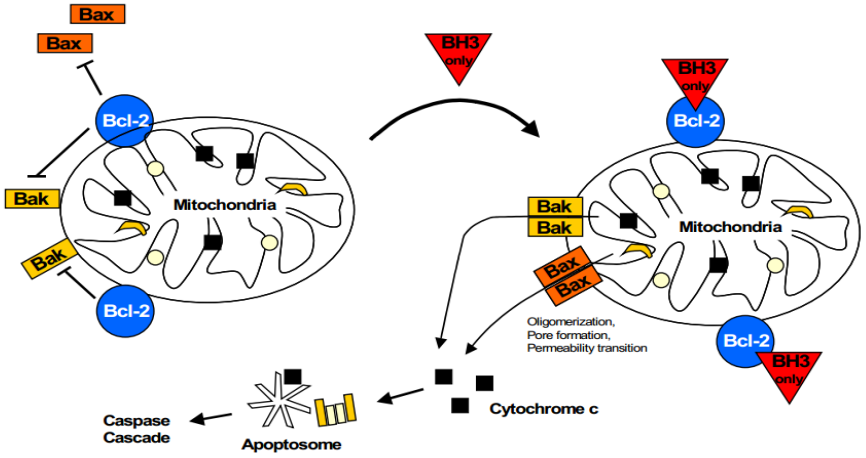


Figure 3.9 Diagram showing the overall functions of Bcl-2 family in apoptotic cell death, taken from Salomon RN *et al* (35).

3.7 Mechanism of caspase cascade activation

Caspases, proteins in cysteine protease family, play crucial parts in apoptosis (37). Caspases are synthesized as inactive proenzyme, called zymogen. Twelve caspases have been discovered in human and they can be classified into initiator caspases and effector caspases. Initiator caspases, i.e. Caspase-2, 8, 9 and 10 make inactive pro-forms of effector caspases become active. The latter are caspase-3, 6, 7 that cleave other protein substrates which are important to trigger the apoptosis (38). Two major pathways; i.e. mitochondria-dependent the intrinsic pathway and extrinsic pathway, involves in caspase cascade of apoptosis.

The death receptor-mediated extrinsic pathway commences through ligand mediated activation of death receptors, such as Fas receptor, tumor necrosis factor on the cell surface (39). Binding of ligand to receptor facilitates oligomerization of the receptors that recruit adaptor molecules and procaspase-8. Afterwards, the death-inducing signaling complex (DISC) (39, 40) is formed, and subsequently DISC activate caspase-8, triggering downstream caspases and apoptosis.

On the other hand, intrinsic pathway is triggered through mitochondria. This pathway is often activated by various apoptosis inducing signals such as loss of cell-survival factors and severe cell stress. In addition, the intrinsic pathway is also

controlled by balance between Bcl-2 family proteins, pro-apoptotic and anti-apoptotic signals. (41) These signals alter the mitochondrial permeability (42), and subsequently facilitate release of cytochrome c. Once cytochrome c release, apoptosome complex formation occurs by recruiting the apaf-1 and procaspase-9 in the cytoplasm. Procaspase-9 is cleaved by apoptosome complex, and activated caspase-9 subsequently stimulates downstream caspases and apoptosis (Figure 3.10).

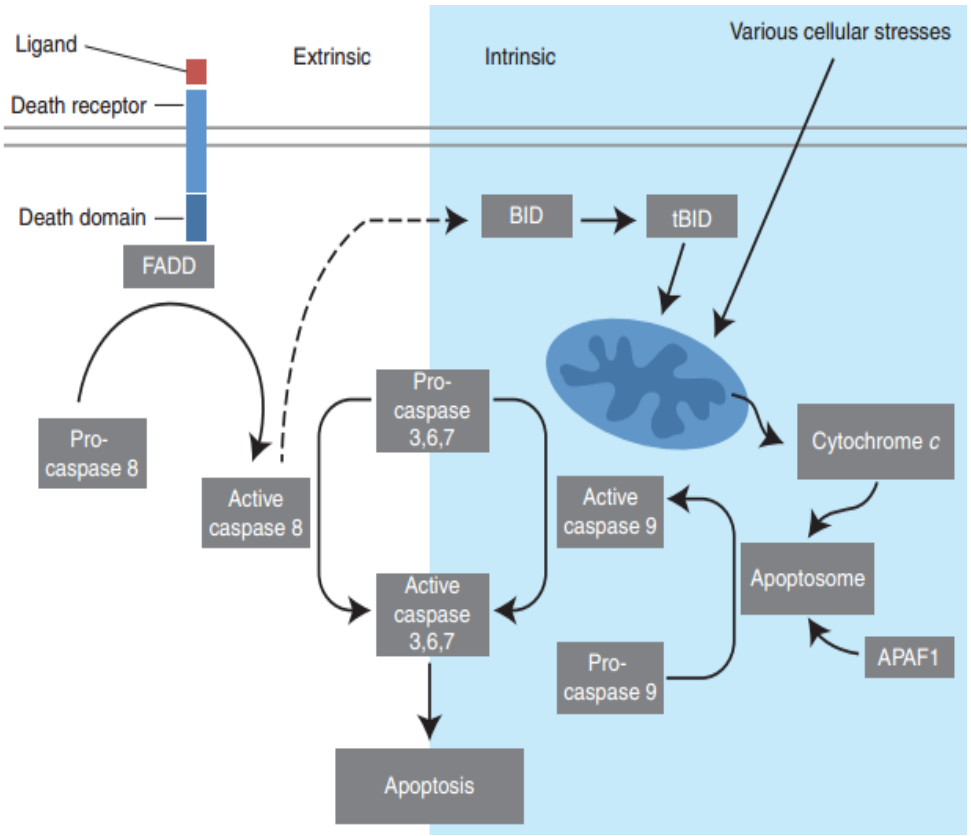


Figure 3.10 Diagram showing caspases cascade mediated by both extrinsic and intrinsic pathway taken from McIlwain DR *et al* (43).

3.8 Apoptosis caused by excess of cellular ROS

Excess intracellular ROS can result in apoptotic cell death through different mechanisms (Figure 3.11). Elevated cellular ROS induces oligomerization of apoptosis signal-regulating kinase 1 (ASK1) rendering a functional ASK1

signalosome complex through ASK1 autophosphorylation. Subsequently, activated ASK 1 causes activation of JNK, c-Jun N-terminal kinases. Nuclear translocation of activated JNK promotes pro-apoptotic genes expression, in which intervention is mediated by activator protein-1 (AP-1) (44). High level of ROS influence mitochondrial permeability transition protein (MPTP) complex and therefore promotes apoptotic cell death. Oxidative modifications of proteins that are components of MPTP decrease mitochondrial membrane potential and cause translocation of Bax and Bad proteins (44). Translocation of these pro-apoptotic proteins lead to release of mitochondrial cytochrome c.

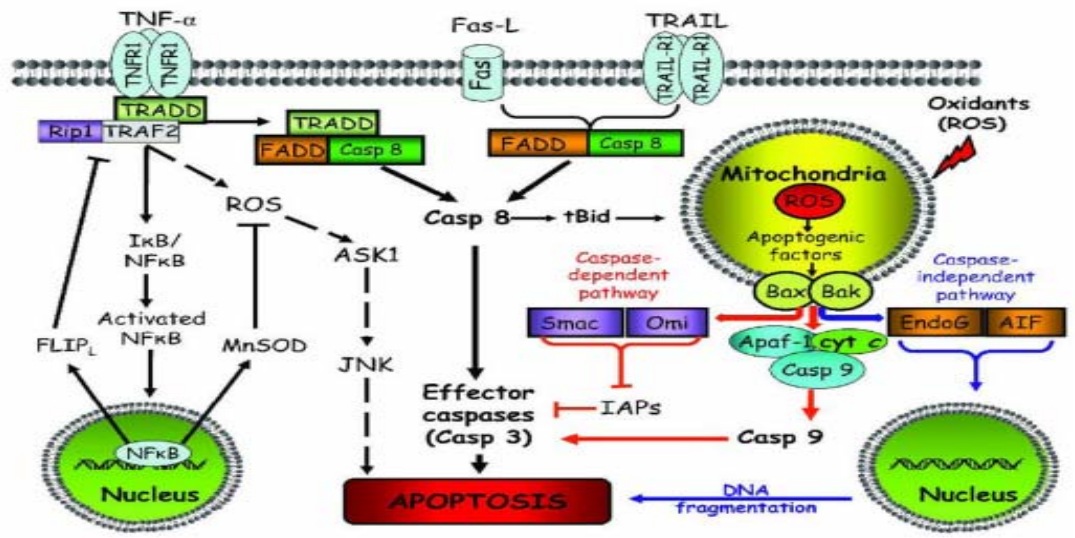


Figure 3.11 Apoptotic cell death pathways caused by excess of intracellular ROS taken from Circu ML et al (44).

3.9 Antioxidant

Antioxidant is a molecule that readily donate its electron to highly reactive ROS, albeit presence at low concentrations. (45, 46). According to solubility, antioxidants are classified into water-soluble antioxidants and fat-soluble group. Ascorbic acid (Vitamin C), glutathione and lipoic acid are water-soluble antioxidants. Polyphenols (e.g. ubiquinone, or coenzyme Q10), α-Tocopherol (Vitamin E), gallates, resveratrol, polyene compounds (e.g. β-Carotene) are fat-soluble antioxidants. On the

other hand, antioxidant can also be classified into enzymatic and non-enzymatic groups as shown in (Figure 3.12).

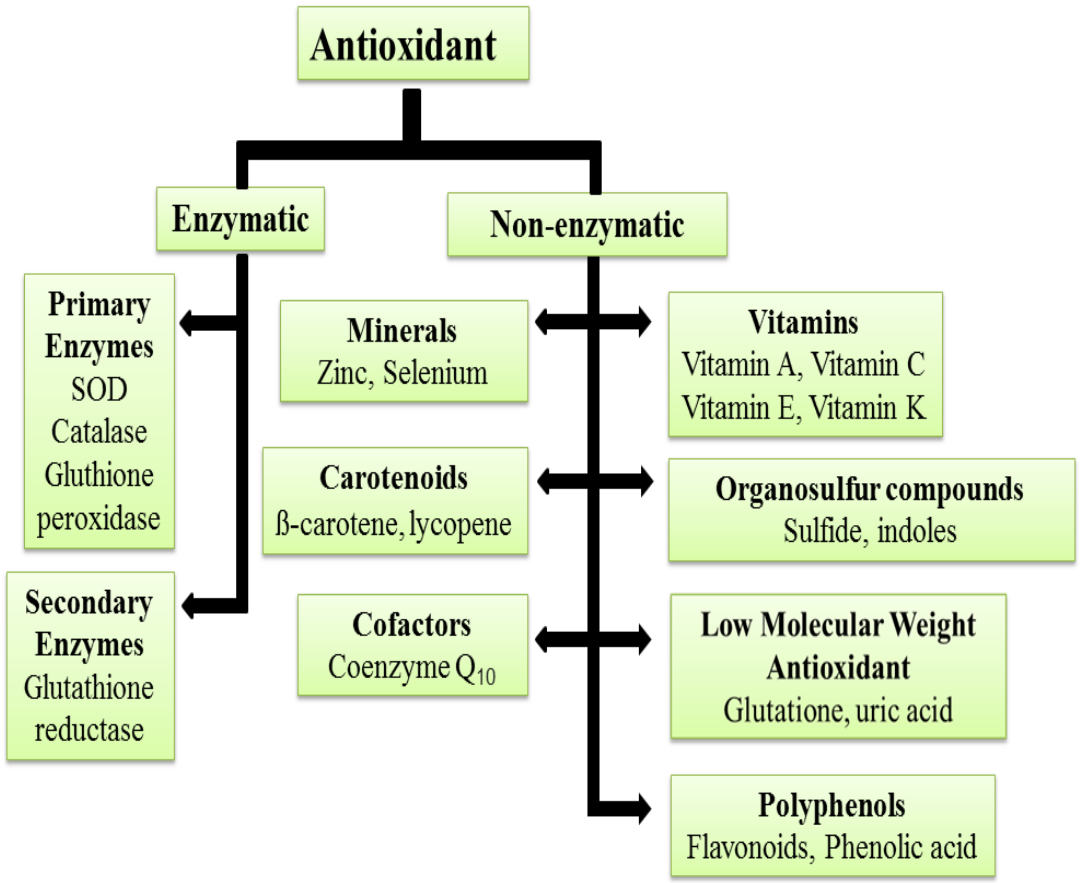


Figure 3.12 Classification of antioxidants, taken from Shalaby EA *et al* (47).

3.10 Antioxidant defense mechanisms

Two antioxidant defense systems; i.e. enzymatic and nonenzymatic systems, are present in the cells of the human body. Enzymatic antioxidants also known as radical scavenging enzymes; i.e. superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), are classified as primary enzymes. The secondary enzymes are glutathione reductase and glucose 6-phosphate dehydrogenase which are synthesized endogenously in the human body (10, 48). Non-enzymatic antioxidants are composed of vitamins, for instance α-tocopherol (vitamin E), ascorbic

acid (vitamin C), vitamin A and vitamin K, minerals such zinc and selenium and carotenoids, glutathione (GSH) and polyphenols.

The family of superoxide dismutase enzymes (SOD) decomposes superoxide radicals into hydrogen peroxide (H_2O_2) which are being neutralized by catalases (CAT), glutathione peroxidase (GPx) through the glutathione (GSH) redox cycle (49, 50) (Figure 3.13). Catalase turns hydrogen peroxide into water and oxygen and it is one of the most important enzymes in protecting the cell (51). In the glutathione (GSH) system, hydrogen peroxide is reduced to water whereas glutathione (GSH) is simultaneously oxidized in a reaction catalyzed by glutathione peroxidase. Oxidized glutathione can be converted into reduced form by glutathione reductase, and glutathione S-transferase can also detoxify the secondary product (Figure 3.13). Imbalance of antioxidant enzyme cofactor, minerals such as selenium and zinc in human body may cause enzymatic antioxidant defense systems dysfunction (52). On the other hand, an effective non-enzymatic antioxidant defense system is also vital to complete the defense system in human body. The most important non-enzymatic lipid soluble antioxidant is vitamin E, an effective chain breaking antioxidant. Vitamin E can also prevent the free radical reaction propagation (53) (Figure 3.13). Moreover, hydrophilic non-enzymatic antioxidant, glutathione and vitamin C play an important role in antioxidant defense system. Vitamin C functions as an electron donor for free radical species (54) and also participates in recycling of α-tocopherol radical (Figure 3.13).

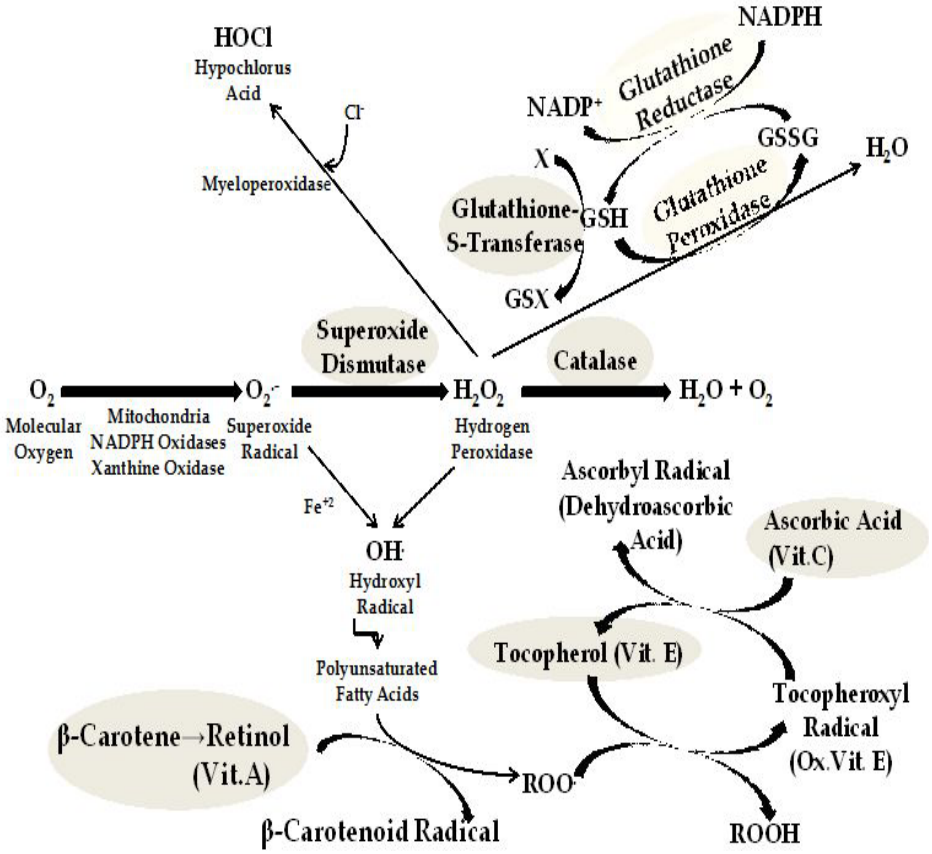


Figure 3.13 Enzymatic and non-enzymatic antioxidant mechanisms, taken from Atukeren P *et al* (55).

3.11 N-trans-feruloyltyramine

N-trans-feruloyltyramine (NTF), a purified extract from local medicinal plant, is called Polyalthia suberosa. (Figure 3.14) it is a polyphenolic compound. Stems of Polyalthia suberosa were collected and extracted by chromatographic separation using hot acetone extract to yield NTF (12). In the chemical structure of NTF, phenolic hydroxyl groups attach to benzene ring (Figure 3.15). NTF has been shown its antioxidant property (13, 14) and the free radical scavenging activity in cells treated with DPPH (1,1-diphenyl-picrylhydrazyl) (56). Phenol groups, attached to benzene rings on NTF compound, scavenged an electron from DPPH (Figure 3.16).

ชื่อ *Thai Name*	กลึงกล่อม
ชื่อวิทยาศาสตร์ *Scientific Name*	*Polyalthia suberosa* (Roxb.) Thwaites
ชื่อวงศ์ *Family*	ANNONACEAE
ชื่อเรียกอื่น *Other Name*	กระทุ่มกลอง กระทุ่มคลอง ขั้งกลอง ท้องคลอง กำจาย ไคร่น้ำ จิงกล่อม ช่องคลอง น้ำนองน้ำน้อย ผักจ้ำ มะจ้ำมงจาม
ลักษณะ *Characteristics*	ไม้พุ่มกึ่งไม้ต้นขนาดเล็ก สูง 2-5 ม. แตกกิ่งต่ำ ใบเดี่ยว เรียงสลับ รูปขอบขนานหรือรูปใบหอกแกมรูปขอบขนาน แผ่นใบเกลี้ยงทั้งสองด้าน หรืออาจมีขนสั้นๆ เหลืออยู่ตามเส้นกลางใบ ดอกออกเดี่ยวๆ ตามด้านข้างของกิ่ง ตรงข้ามหรือเยื้องกับใบ หรือออกเหนือง่ามใบเล็กน้อย ก้านดอกเรียว กลีบเลี้ยง 3 กลีบ รูปไข่ ด้านนอกมีขน กลีบดอกสีเหลืองอมน้ำตาล เรียงสลับกัน 2 ชั้น ชั้นละ 3 กลีบ ขนาดใหญ่กว่ากลีบเลี้ยง เกสรเพศผู้เล็ก มีจำนวนมาก อยู่ชิดกันแน่นเป็นพุ่มกลม ผลเป็นผลกลุ่มมีจำนวนมาก อยู่บนแกนตุ้มกลม แต่ละผลกลม ผิวเรียบ สีเขียว เมื่อสุกสีแดง มี 1-2 เมล็ด
การกระจายพันธุ์ *Distribution*	อินเดีย ศรีลังกา จีน ลาว พม่า ไทย เวียดนาม มาเลเซีย และฟิลิปปินส์ พบตามป่าเบิด ที่ระดับความสูงไม่มาก

Figure 3.14 Botanical background of Polyalthia suberosa, taken from BGO Plant Database, The Botanical Garden Organization (www.qsbg.org)

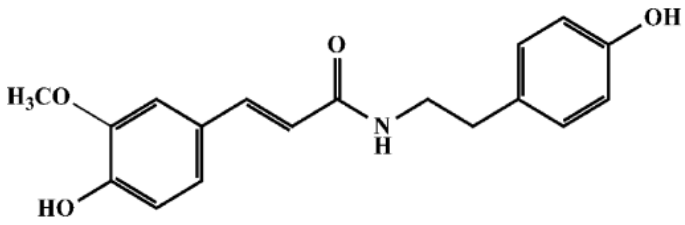


Figure 3.15 Chemical structure of N-trans-feruloyltyramine. Taken from chemblink http://www.chemblink.com/

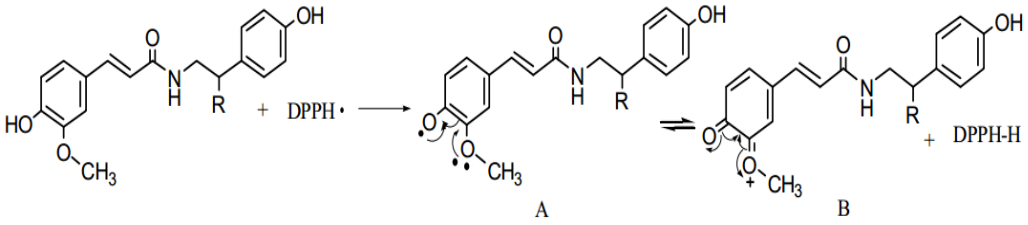


Figure 3.16 Propose model showing the free radical scavenging activity of NTF, taken from Li WJ et al (56).

CHAPTER IV
MATERIALS AND METHODS

4.1 SK-N-SH cells culture

SK-N-SHs are cultured human neuroblastoma cells (ATCC, Rockville, MD, USA), and they were proliferated in a humidified incubator with 5% CO_2 at 37℃, and maintained in MEM medium with 10% (v/v) fetal bovine serum, streptomycin (100ug/ml), and penicillin G (100 units/ml). Cells were detached by 0.25% trypsin EDTA (Gibco). Afterwards**,** cells were resuspended with fresh media and centrifuged at 2,000 g for 3 minutes. Cell pellet was dissociated and distributed evenly in cultured flask. Cells were propagated to 80 to 90% of culturing space. Suitable numbers of cells were plated for further experiments.

4.2 Preparation of *N-trans*-feruloyltyramine

N-trans-Feruloyltyramine (NTF), extracted from a local medicinal tree, called *Polyalthia suberosa,* was kindly provided by Dr Patoomratana Tuchinda, Dr Bamroong Munyoo and Dr. Saksit Nobsathian; Department of Chemistry, Faculty of Science, Mahidol University. Stems of *P. suberosa* were collected from Kalasin Province in the northeastern part of Thailand and extracted by chromatographic separation using hot acetone extract to yield NTF (Tuchinda et al., 2000).

4.3 Experimental designs and procedures

4.3.1 Optimization for hydrogen peroxide concentration on cell viability

Hydrogen peroxide at various concentrations (37.5 to 2400 µM) was used to confirm the cytotoxicity of H_2O_2 on cultured SK-N-SH. $2x10^4$cells/100µl were plated in 96 well plates. H_2O_2 (Merck Schuchardt) is freshly diluted in phosphate buffer saline solution prior to adding onto the culture at designated final concentrations (37.5-2400 µM). The cell viability was determined after 24 hours incubation by using MTT assay. No H_2O_2 treatment group was used as a control.

4.3.2 Test for *N-trans*-feruloyltyramine toxicity on cell viability

NTF was pre-incubated on SK-N-SH to assess its toxicity. $2x10^4$cells/100µl were plated in 96 well plates and NTFs (10 to 500 µM) were added to the cultures and incubated for 3 hours. After that the cell viability was determined by using MTT assay. No NTF treatment group was used as a control.

4.3.3 Assessment for the protective role of *N-trans*-feruloyltyramine in hydrogen peroxide induced SK-N-SH cell death

Experimental groups

Group 1 – without H_2O_2

Group 2 – with H_2O_2

Group 3 –pretreated NTF+ H_2O_2

To determine the protective effect of NTF on cell viability, $2x10^4$cells/100µl were plated in 96 well plates. NTFs at designated concentrations (10 to 500 µM) were used to pre-incubated for 3 hours prior to 24 hours period of H_2O_2 insult at 150 µM. MTT assay, ROS level, caspase-3 activity and cellular morphology were assessed and activated caspase-3 and pro-apoptotic protein, Bax were determined by western blotting.

4.4 MTT assay

Spectrophotometric analysis of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was used to determine viability of the cells (57, 58). Increased crystalline blue formazan reflected cell viability (Figure 4.1). Once being dissolved, blue formazan will be analysed at 570 nm by microplate reader (Molecular Device). In the experiment of MTT assay, 5mg/ml of MTT solutions in PBS was used and 20µl/well was added into each well. After 3 hours incubation, MTT solutions were discarded and formazan crystals were dissolved with dimethyl sulfoxide (DMSO). Microplate reader measured the signal intensity at a wavelength of 570 nm. The results were reported as the percentage of viable cells compared to control.

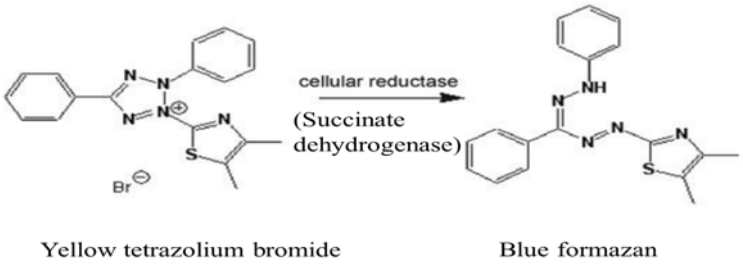


Figure 4.1 Conversion of yellow tetrazolium bromide to a crystalline blue formazan by cellular enzyme (succinate dehydrogenase)

4.5 Assessment of cellular morphology

Cellular morphological changes were determined by phase contrast inverted microscope. Experimental protocol is shown in Figure 4.2.

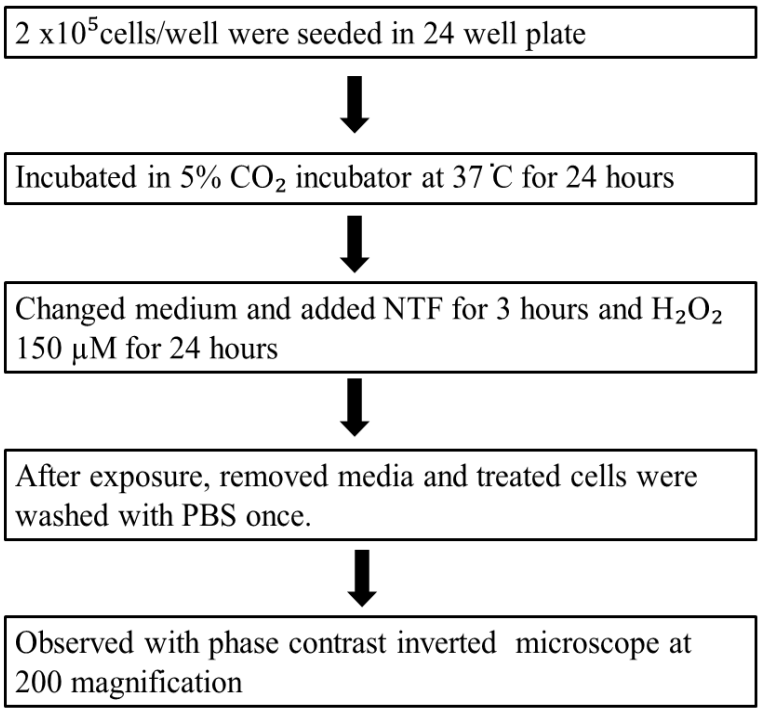


Figure 4.2 Experimental procedure to determine cellular morphology.

4.6. Assay for Level of Cellular Reactive Oxygen Species (ROS)

Levels of intracellular ROS were assessed using fluorescein probe, 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) (59, 60). Cellular esterases deacetylated DCFH-DA to form non-fluorescent compound, DCFH. ROS oxidize DCFH, as a result, highly fluorescent molecules (DCF) emit fluorescent signal proportionate to intracellular ROS. (Figure 4.3) In the ROS assay experiment, cells were incubated in the dark with 10 µM DCFH-DA at 37C. After 30 minutes, DCFH-DA solution was removed and rinsed once with PBS. NTF at the concentrations of 25, 50,100 µM were pre-incubated and 150 µM of H_2O_2 was added thereafter, as described above. Fluorescent signal was quantified by microplate reader (BioTek Instrument) at excitation and emission wavelength of 485/530 nm. The results were reported as the percentage of viable cells compared to control.

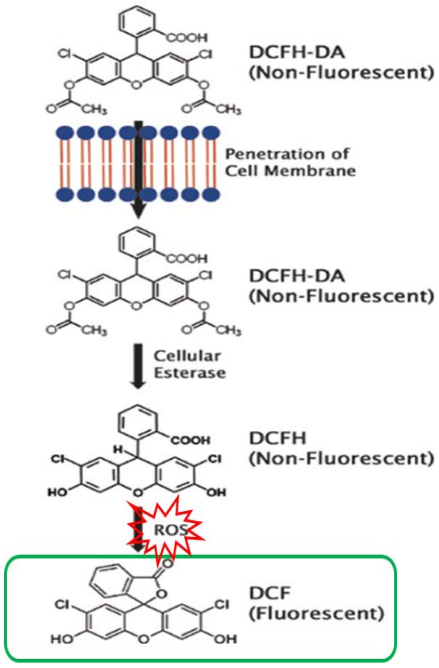


Figure 4.3 Formation of fluorescent (DCF) proportionate to intracellular ROS.

4.7. Assessment of caspase-3 activity

CaspACE assay system (Promega), a colorimetric assay kit, was used to measure the caspase-3 activity. Chromophore p-nitroanilline (pNA) was released after specific cleavage of caspase-3, and measured by microplate reader at the wavelength of 405 nm. Experimental procedure to determine caspase 3 activity is described in Figure 4.5.

Figure 4.4 Release of pNA from the substrate upon a cleavage by caspase-3

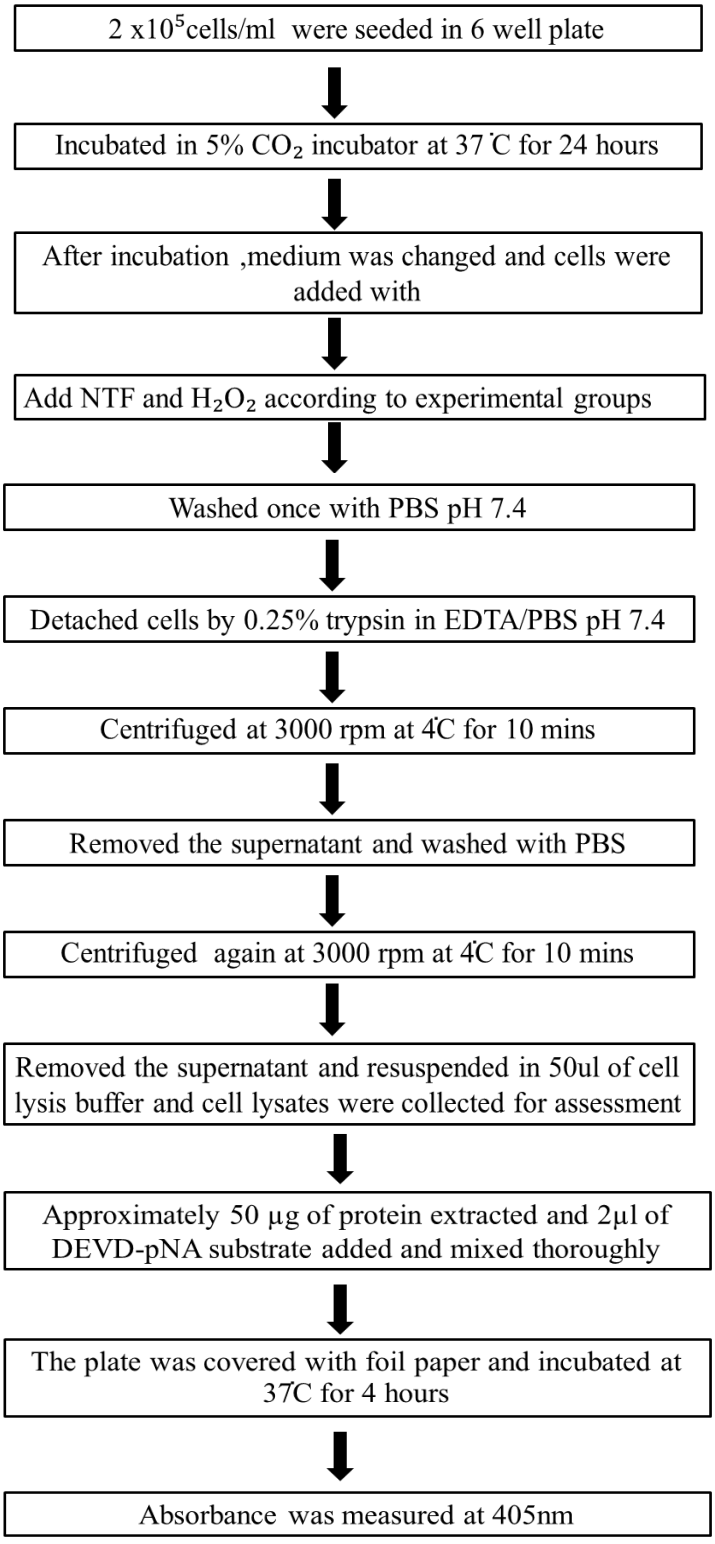


Figure 4.5 Experimental procedures to assess caspase-3 activity

4.8 Analysis of Caspase-3 and Bax levels by immunoblotting

Pro-apoptotic protein Bax and caspase-3 level were analysed by immunobloting. Equal amounts of protein samples (measured by Nano Photometer, IMPLEM) were fractionated by 10% SDS Polyacrylamide gel electrophoresis (PAGE) in Tris Boric EDTA buffer at 100 V for 1hour and 45mins. The fractionated proteins were transferred onto a polyvinyl membrane (Biorad) at 70 V for 2 hours at 4◦C. 5% bovine serum albumin (BSA) were used for blocking the membrane for 1 hour. Antibody detecting human actin and activated caspase-3 from Cell Signaling), antibody detecting human Bax from Santa Cruz were used for protein detection, and secondary antibodies conjugated with horseradish peroxidase (HRP) were be incubated at room temperature for 2 hours. Protein bands were detected using enhanced chemiluminescent reagents, Clarity™ Western ECL Substrate (BIO-RAD). ImageJ densitometry software was used to detect and measured the band density.

4.9 Statistical Analysis

Statistical analysis for all experiments was analysed by one-way analysis of variance (ANOVA), and the Student–Newman–Keul's was undertaken to confirm post ANOVA. Data were calculated using Graphpad Prism software version 5. All data were presented as 3 independent experiments with standard error of the mean.

4.10 Chemicals and Reagents

Acrylamide (Pharmacia)

Ammonium persulfate (Gibco BRL)

Bromophenol blue (Sigma)

Bovine serum albumin (Sigma)

CaspaseACE assay system (Promega)

Clarity™ Western ECL Substrate (BIO-RAD)

DMSO (Sigma)

DCFH-DA (Sigma)

Ethylenediamine tetraacetic acid, EDTA (Sigma)

Fetal bovine serum (Biochrom)

Fungizone (Bristol-Myers Squibb)

Hydrogen peroxide (Merck)

L-glutamine (Biochrom)

MEM medium (Invitrogen)

MTT (Sigma)

Mouse primary antibody against human Bax (Santa Cruz)

Mouse primary antibody against human actin (Cell Signaling)

Rabbit primary antibody against activated human caspase-3 (Cell Signaling)

90% methanol (Merck)

Non essential amino acid (Biochrom)

Penicillin G (M & H)

Sodium pyruvate (Sigma)

Sodium bicarbonate (Merck)

Sodium dodecyl sulphate (SDS) (Merck)

Streptomycin (M & H)

TEMED (Pharmacia)

Trypsin (Biochrom)

Tris (hydrochloride) (Sigma)

Tween-20 (BIO-RAD)

4.11 Equipment

Analytical Balance, AG20 4 DR

Autoclave, HL-341, Glovia, Taiwan LTD

Automatic Adjustable micropipettes

Basic pH meter, Orion 410A^{+}Thermo Electron Corporation

CO_2 incubator, Sanyo Electric Co,ltd, Japan.

Electrophoresis Power supply (BIO-RAD)

High Speed centrifuge, Thermo scientific Germany

Hemacytometer

Inverted Light microscope (Olympus)

Laminar Flow

Waterbath (Imperial II)

Vortex Mixer (Scientific Indus Inc)

Refrigerator 4℃ (Panasonic)

Deep Freezer -20 ℃ (Panasonic)

Microcentrifuge

Microplate reader (Bio-Tex EL 311)

Water purification equipment (Millipore)

Eppendorf micro tubes (Corning Incorporated, USA)

Culture cluster (Corning Incorporated, USA)

Centrifuge tube (Corning Incorporated, USA)

Serological pipette (HBG,cGermany)

Volumetric Flask (Schott)

CHAPTER V
RESULTS

5.1 Optimization for H_2O_2 Concentration on SK-N-SH Cell Viability

H_2O_2 treatments at different concentrations on cultured SK-N-SHs were assessed their viability at 24 hour after incubation. The result indicated that H_2O_2 can induce SK-N-SH cells death in a dose dependent manner (Figure 5.1). Percentage of Cell viability compared to control was expressed as mean with standard error of mean of three independent experiments. The percentage of cell viability from cells treated with H_2O_2 at 0, 37.5, 75, 150, 300, 600, 1200 and 2400 μM showed that 85.12±2.04, 79.46±1.01, 58.04±6.77, 48.13±6.68, 36.65±6.66, 28.69±2.87, 23.83±3.53 respectively. Newman-Keuls multiple comparison test showed statistical significances at all tested concentrations. H_2O_2 treatment at the concentration of 150 μM was chosen for subsequent experiments, as this concentration of hydrogen peroxide led to approximately 50% cell death.

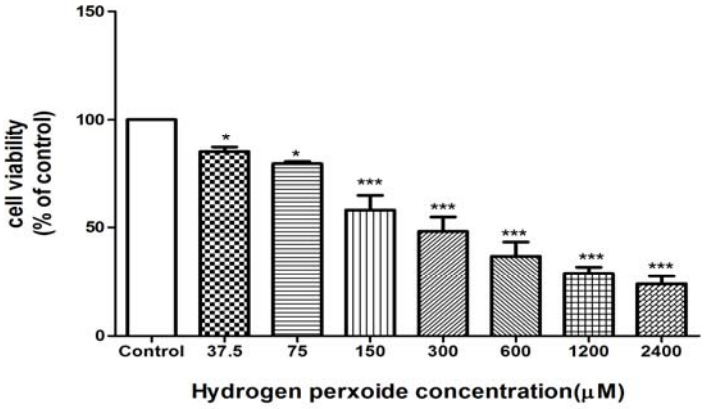


Figure 5.1 Viability of cultured SK-N-SHs after treatment of H_2O_2 at various concentrations, as indicated. The percentages of the cell viability from different concentrations of H_2O_2 were presented as mean with standard error of mean of three independent experiments. (*$p < 0.05$, ***$p < 0.001$ compared to untreated control).

5.2 Effect of NTF on Cell Viability

Various concentrations of NTFs (10, 25, 50, 100, 150, 250, 500 μM) were used to assess toxicity of this compound. Cell viability was expressed as mean of percentage with standard error of the mean derived from three independent experiments. The percentage of cell viability from NTF treatment at the concentrations of 10, 25, 50, 100, 150, 250 and 500 μM resulted in 99.44±0.94, 92.89±1.90, 94.27±0.74, 93.70±1.99, 94.72±1.90, 92.89±2.85, 96.34±2.62 respectively. Notably, increased concentrations of NTF were not imposed any observable toxicity on the cells (Figure 5.2).

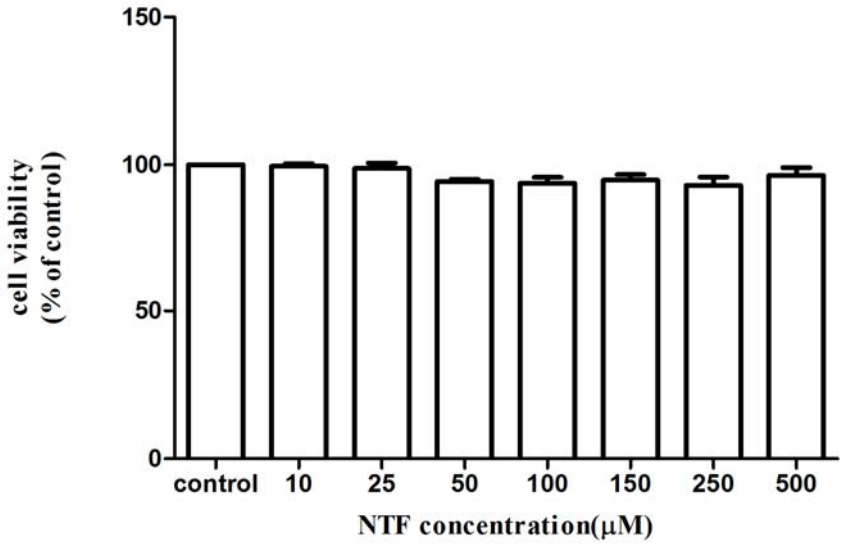


Figure 5.2 Viability of SK-N-SH cells after treatment of NTF at various concentrations, as indicated. There is no significantly toxic effect to the cell viabilities at all concentrations.

5.3 Apoptosis induced by H_2O_2 can be prevented by NTF treatment

NTFs at various concentrations (10, 25, 50, 100, 150, 250 and 500 μM) were used to demonstrate its protective effect on hydrogen peroxide insulted SK-N-SH cell. The percentage of cell viability from SK-N-SH cells treated with NTF at the concentrations of 10, 25, 50, 100, 150, 250 and 500 μM resulted in 52.62±3.06, 79.85±0.70, 82.00±1.48, 82.41±2.68, 84.78±.07, 84.54±0.52, 86.32±2.09 respectively.

Viability of cells insulted with H_2O_2 was substantially reduced compared to untreated control and this effect was preventable by NTF treatment at all tested concentrations (figure 5.3)

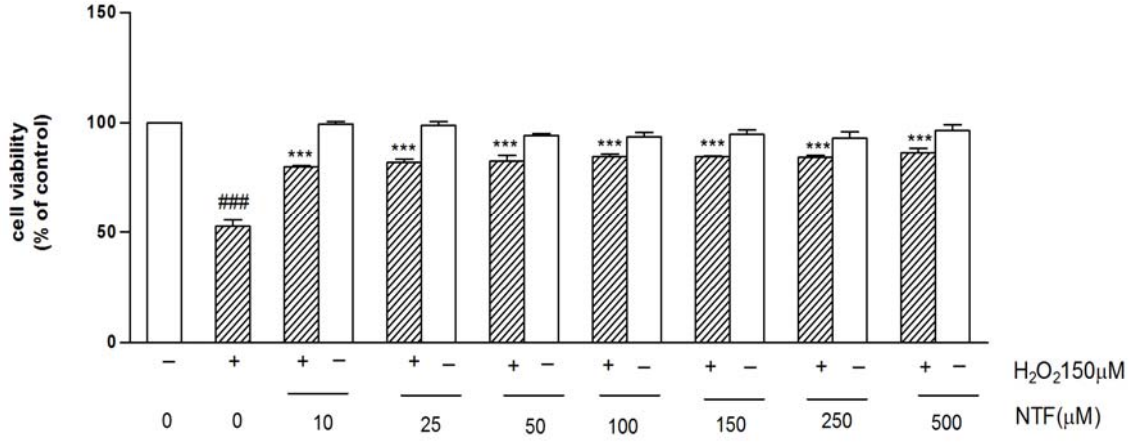
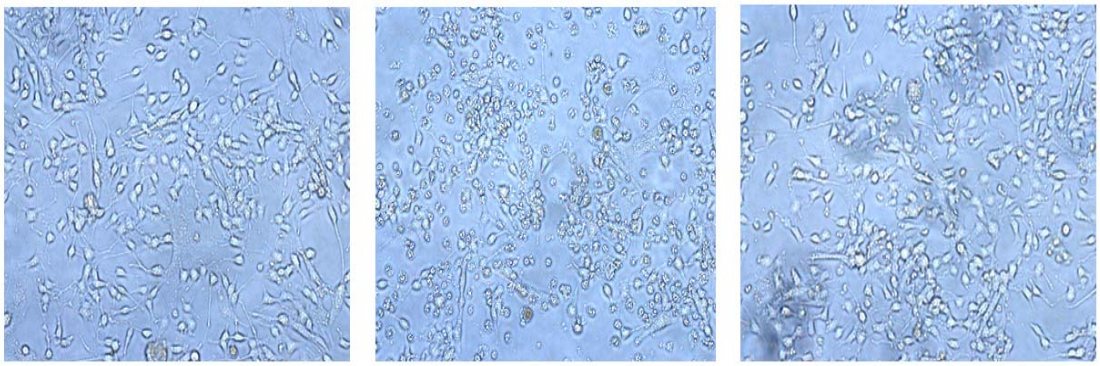


Figure 5.3 Protective effects of NTF on H_2O_2-induced toxicity in cultured SK-N-SH cells. Results are presented as mean with standard error of mean of three independent experiments. (### $p < 0.001$ compared to control and ***$p < 0.001$ compared to H_2O_2 -induced group)

5.4 Assessment of cellular morphology

At 150 μM of H_2O_2 insult, SK-N-SH cells became shrinkage, detached from the bottom of the well and formed cell aggregations (Figure 5.4B). NTF at the concentration of 50 μM attenuated H_2O_2-induced morphological alterations (Figure 5.4C).



(A) (B) (C)

Figure 5.4 Cellular morphology of untreated control (A), cells induced with 150 μM H_2O_2 of (B), cells pre-treated with 50 μM of NTF and following by 150 μM of H_2O_2 (C).

5.5 ROS induction by H_2O_2 can be scavenged by NTF

Level of intracellular ROS was assessed to detect the antioxidative effect of NTF in SK-N-SH cells insulted with H_2O_2. Percentage of DCF fluorescence was expressed as mean with standard error of mean of three independent experiments. ROS production significantly increased after treatment of H_2O_2 (150 μM). At all three concentrations (25, 50, 100 μM) of NTF pre-incubation, intracellular ROS was significantly reduced compared to hydrogen peroxide treated group. (Figure 5.5), and scavenging effect of NTF was not only for induced intracellular ROS but also for endogenous intracellular ROS production.

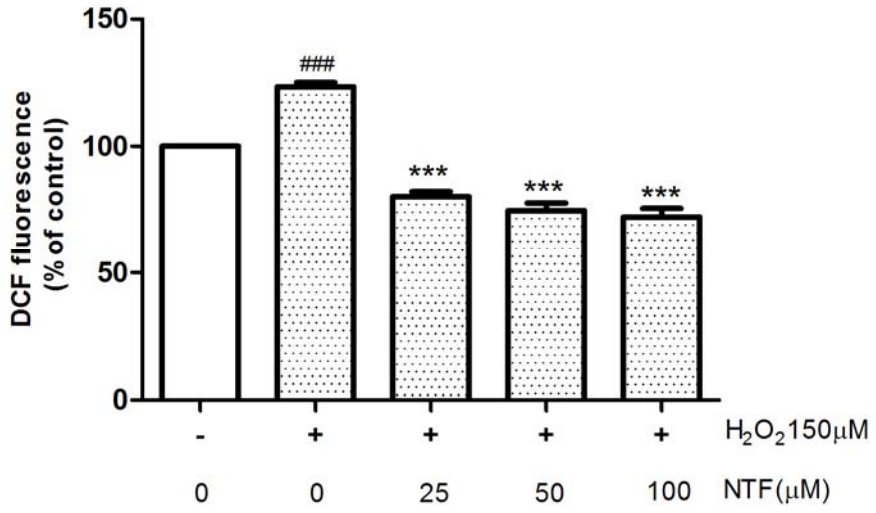


Figure 5.5 Levels of intracellular ROS were significantly decreased by NTF pre-incubation. Results were presented as mean with standard error of the mean of three independent experiments. (### $p< 0.001$ compared to control and, *** $p< 0.001$ compared to H_2O_2-induced group)

5.6 Analysis of pro-apoptotic protein, Bax by immunoblotting

Pro-apoptotic protein, Bax was significantly enhanced after treatment of 150 μM of H_2O_2. NTF at 25 μM concentration can significantly prevent increased Bax expression; however to a higher extent, NTF at the concentrations of 50 and 100 μM can normalize induced expression of Bax to the level that is comparable to no hydrogen peroxide control (Figure 5.6). Relative level of Bax in percentage of control was expressed as mean with standard error of mean of three independent experiments.

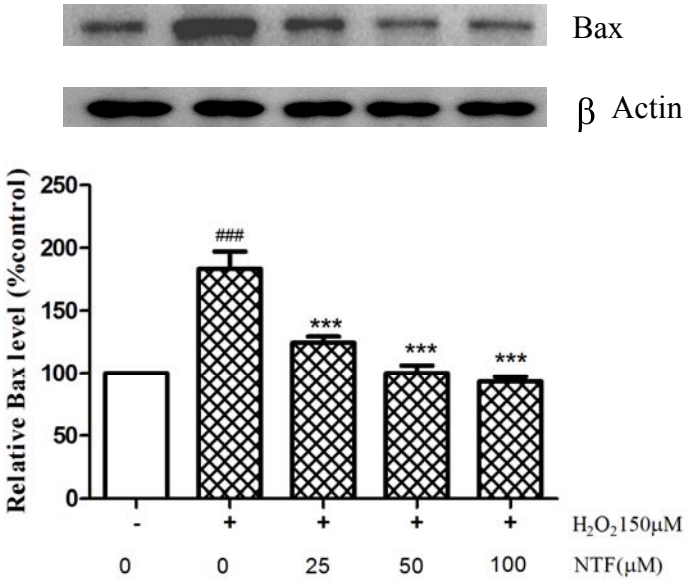


Figure 5.6 Western blot analysis of pro-apoptotic protein Bax in SK-N-SH cell line. NTF protects SK-N-SH cells insulted with H_2O_2. Pretreatment of NTF 25, 50 and 100 μM significantly reduces expression of Bax. Results are expressed as mean with standard error of the mean of three independent experiments (### p< 0.001 compared to control and, *** p< 0.001 compared to H_2O_2-induced group)

5.7 Analysis of activated caspase-3 levels by Immunoblotting

Activated caspase-3 level was significantly increased after treatment of H_2O_2 at the concentration of 150 μM, while NTF at all tested concentrations (25, 50 and 100 μM) can normalize induced expression of activated caspase 3 to the level that is comparable to no hydrogen peroxide control (Figure 5.7).

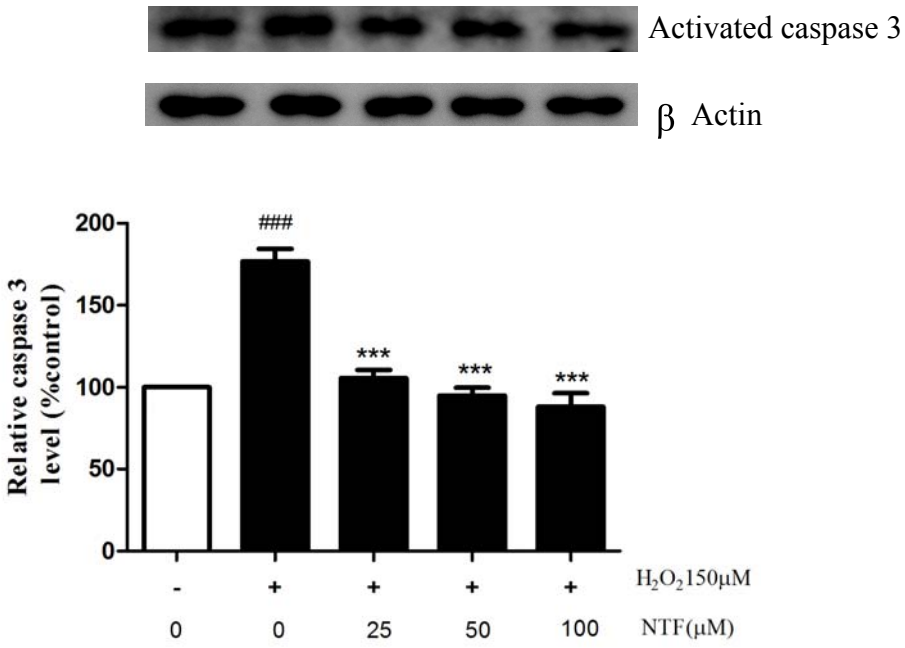


Figure 5.7 NTF protects SK-N-SH cell death against H_2O_2 induced apoptosis. All pretreated concentration (25, 50 and 100 μM) of NTF significantly decreases activated caspase-3 level. Results are expressed as mean with standard error of mean of three independent experiments. (### p< 0.001 compared to control and, *** p< 0.001 compared to H_2O_2-induced group)

5.8 Determination of caspase-3 activity

CaspACE assay was used to measure the activity of caspase-3 and it was expressed as mean with standard error of mean of three independent experiments. Significant increase in caspase-3 activity was observed after treatment of 150 μM of H_2O_2. Caspase-3 activity from SK-N-SH cultures treated with 150 μM of H_2O_2 was approximately four-fold increase compared with control and pretreatment of NTF significantly decreased activity of caspase-3 at all treated concentrations 25, 50 and 100 μM. (Figure 5.8)

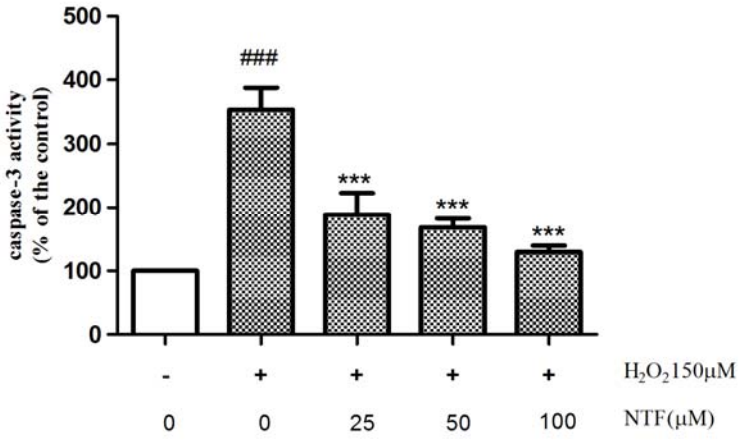
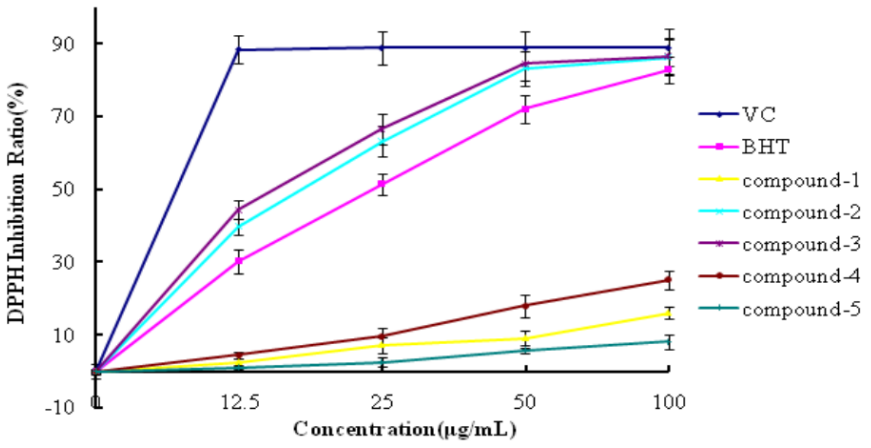


Figure 5.8 Activity of caspase-3 was measured in untreated cells and SK-N-SH cells treated with various concentration of NTF in presence of H_2O_2. Data were presented as percentage of the activity as mean of three independent experiments (### p< 0.001 compared to control and, *** p< 0.001 compared to H_2O_2-induced group)

CHAPTER VI
DISCUSSION

6.1 Antioxidant activity

N-trans-feruloyltyramine (NTF), a plant polyphenolic compound, has been shown for its antioxidant property and the free radical scavenging activity (12, 13). Interestingly, the previous study reported that the chemical structure of NTF, phenolic hydroxyl group, can scavenge the electron from stable free radical DPPH (53). In this study, antioxidant activity of NTF have confirmed in an *in vitro* cell model by demonstrating that NTF could prevent cytotoxicity in an oxidative stress cell model through scavenging intracellular ROS. In addition, we also further investigated that the antioxidant effect of NTF can neutralized intracellular ROS and are sufficient to prevent cell death in SK-N-SH cells under oxidative stress conditions. In this study, we did not perform a direct comparison between NFT and other antioxidants; however Wen Jie Li *et al* (56) reported how well five phenolic compounds can scavenge the DPPH radicals compared with reference antioxidants. NTF was more effective antioxidant compared to other tested phenolic compounds, and the scavenging activity of NTF is comparable to vitamin c. (Figure 6.1)



VC (vitamin C)

BHT (butylated hydroxytoluene)

Compound -1 (N-trans-coumaroyltyramine)

Compound -2 (N-trans-feruloyltyramine)

Compound -3 (N-trans-feruloyloctopamine)

Compound -4 (5, 7-dihydroxy-8-methoxyflavone)

Compound -5 (3, 5, 4′-trihydroxy-7-methoxy-6-methylhomoiso-flavonone)

Figure 6.1 Scavenging activity of NTF compared with other compound and references antioxidants taken from Wen Jie Li et al (56)

6.2 Anti-apoptotic activity

Excess in intracellular ROS results in oxidative stress and can induce cellular apoptosis. High level of ROS causes oxidative modifications of mitochondrial permeability transition protein (MPTP) complex and, in turn, leads to decreased mitochondrial membrane potential. Deranged mitochondrial membrane potential causes translocation of Bax and Bad proteins from the cytoplasm to mitochondria and facilitates apoptosis (44). Translocations of these pro-apoptotic proteins initiate a megapore channel leading to release of mitochondrial cytochrome c. In the cytosol, cytochrome c interacts with procaspase-9 and apoptotic protease-activating factor 1 (apaf-1) to assemble apoptosome complex, thus leading to proteolytic activation of pro-caspase-9. Subsequently, activated caspase-9 promotes activation of caspase-3 and eventually causes cellular apoptosis. In addition, increased level of cellular ROS facilitates ASK1 oligomerization and form functional signalosome complex. Afterward, activated ASK1 cause activation of JNK, thereby leading to nuclear translocation of activated JNK1. Nuclear JNK1 promotes transcriptional activation of pro-apoptotic proteins; i.e. TNFα, Bax and Bak, through activator protein 1 (AP-1) mediated process (61).

In this study, cellular apoptosis caused by H_2O_2 induction, as manifested by the expression of apoptotic proteins, Bax, and activation of caspase-3, a key enzyme involving in cellular apoptosis. NTF could prevent H_2O_2-induced apoptosis by

attenuating the level of caspase-3, Bax and the activity of caspase-3, and NTF can recuperate up to 50% cell death in our *in vitro* model of oxidative stress while NTF treatment on SK-N-SH cells do not induce cell death at any tested concentrations.

6.3 Future approach

NTF could potentially be a good candidate for antioxidant compound to prevent diseases whose oxidative stress plays a critical role in disease pathophysiology. Future studies on the protective effect of NTF should investigate whether this compound would show similar antioxidant effect in relevant animal models of oxidative stress. Assessing both toxicity and effectiveness of NTF in the animal models should be conducted. In addition, the disease animal model that the main etiology of the disease is due to the oxidative stress such as Alzheimer disease should use to test the protective role of NTF. If proven effective, this compound can then proceed to examine potential benefit in clinical trial.

CHAPTER VII
CONCLUSION

In conclusion, this present study was designed to evaluate antioxidant effects of NTF in an *in vitro* cell model (SK-N-SH cells), insulted with H_2O_2 and whether the reduction in ROS level can lead to cytoprotection in our model. Then, the protective effect of NTF was investigated by assessing cell viability using MTT assay, ROS level and morphological changes. Increased levels of proapoptotic protein (Bax) and activities of key enzyme relating to apoptosis, caspase-3 were utilized as a marker for cellular apoptosis. While insulted with 150μM of H_2O_2, SK-N-SH cells resulted in increased ROS level, induced cytotoxic changes and decreased cell viability. On the other hand, preincubation with various concentrations of NTF in our model significantly reduced cellular ROS and attenuated such cytotoxic cell death. In addition, pro-apoptotic protein, Bax expression and relative level of activated caspase-3 significantly decreased at NTF pre-treated cells. Moreover, the H_2O_2-induced caspase-3 activity was also preventable by NTF pretreatment. Therefore, the findings from these experiments showed the antioxidant effect of NTF in an *in vitro* cell model and demonstrated that NTF could successfully reduce cellular ROS and protect against H_2O_2-mediated cytotoxicity and cell death in SK-N-SH cells.

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APPENDIX

Chemicals and Reagents

(1) 30% Acarylamide / 0.8% bis-acrylamide

Acrylamide	6 g
Bis-acrylamide	0.16 g
Deionized water	20 ml

(2) 4X Tris-Cl / SDS pH 8.8 (1.5M Tris-HCl containing 0.4% SDS)

Trisma-base	18.2 g
SDS	0.4 g
Deionized water	60 ml

adjust pH 8.8 and make volume to 100 ml

(3) 4X Tris-Cl / SDS pH 6.8 (0.5M Tris-HCL containing 0.4% SDS)

Trisma-base	3.025 g
SDS	0.2 g
DW	20 ml

adjust pH 8.3 and make volume to 50 ml

(4) 1X SDS / electrophoresis buffer pH 8.3

Trisma-base	3.02 g
Glycine	14.4 g
SDS	1 g

adjust pH 8.3 and make volume to 1000 ml

(5) 1X Transfer buffer pH 8.3

Trisma-base	3.03 g
Glycine	14.4 g
Methanol	120 ml

make volume to 1000 ml with DW

(6) 10 % (w/v) Sodium Dodecyl Sulphate

10 grams of sodium dodecyl sulphate (SDS) was dissolved and adjusted the final volume to 100 ml with Distilled water.

(7) Ammonium Persulphate (10%, w/v)

Ammonium persulphate	1 g
ddH_2O	10 ml

(8) Phosphate buffer saline-Tween 20 (PBST)

1 X PBS	1 L
Tween-20	0.5 ml
(prepared freshly)	

(9) 5 % BSA (Bovine Serum Albumin)

BSA	5 g
PBST	100 ml

(10) Phosphate buffer saline (PBS) pH 7.4

NaCl	8 g
KCL	0.2 g
Na_2HPO_4	1.44 g
$KH_2 HPO_4$	0.2 g

Final volume was adjusted to 1 L using Deionized water and stored at room temperature.

BIOGRAPHY

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