# EFFECT OF OXIDIZED LOW DENSITY LIPOPROTEIN ON NEURONAL CELLS DYSFUNCTION LINKED TO ALZHEIMER'S DISEASE

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## ABSTRACT

Hypercholesterolemia is widely recognized as one of the risk factors for cardiovascular diseases and is believed to be associated with risk of Alzheimer's disease. The present study aimed to investigate the effect of two oxidized LDL preparations; mildly oxidized (mox-LDL) and fully oxidized-LDL (fox-LDL) in comparison with native LDL. Their effect on cell viability, intracellular ROS production, and AChE activity and expression were determined in human SH-SY5Y neuroblastoma cells. Necrotic and apoptotic death, were also determined by LDH release and caspase-3 activity, respectively. At subtoxic concentration of oxidized LDL, pathways of APP processing were explored by monitoring the levels of released sAPPβ and sAPPα in culture medium and BACE-1 activity in cell lysate. Intracellular signaling, the phosphorylation of CREB transcription was also determined. The results demonstrated that oxidized LDLs possessed neurotoxicity in dose- and time-dependent patterns that potentially resulted from the increased intracellular reactive oxygen species (ROS) production. Oxidized LDL increased the activity of cellular AChE, and this increment was correlated with intra cellular ROS levels. Highly oxidized LDL caused necrotic cell damage, whereas mox-LDL containing low level of oxidized lipid led to apoptotic death. Interestingly, only native LDL promoted APP processing to amyloidogenic pathway without affecting total APP expression in neurons. Oxidized

LDL herein failed to induce the release of APP but enhanced cellular AChE activity. BACE-1 activity was not changed by all LDL sample preparations. In addition, native LDL or mox-LDL acutely increased CREB phosphorylation and this phenomenon was decrease at 1 h. On the other hand, fox-LDL constantly reduced CREB phosphorylation. The reduction in CREB phosphorylation might be consequently the loss of neuronal plasticity. Our study suggests that native LDL and oxidized LDL promoted AD pathogenesis via different pathways. Elevated serum LDL possibly involves in the development of AD. Under the circumstance of oxidative stress, the formation of oxidized LDL may aggravate the progression of AD pathological processes in hypercholesterolemia. Maintaining LDL level in the circulation and balancing between anti-oxidant and oxidation may be potential strategies to prevent or delay AD neurodegeneration.

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# ABBREVIATIONS

| | | |
|---|---|---|
| 24-OHC | = | 24S-hydroxycholesterol |
| 27-OHC | = | 27-hydroxycholesterol |
| Aβ | = | amyloid β-peptide |
| AD | = | alzheimer's disease |
| AChE | = | acetylcholinesterase |
| APP | = | amyloid precursor protein |
| ApoB-100 | = | apolipoprotein B100 |
| ApoE | = | apolipoprotein E |
| AMC | = | 7-amino-4-methylcoumarin |
| BACE-1 | = | β-site APP cleaving enzyme |
| BBB | = | blood-brain barrier |
| BCA | = | bicinchoninic acid assay |
| ° C | = | degree celcius |
| CaMK | = | Ca2+/ Calmodulin-dependent protein kinase |
| $CO_2$ | = | carbondioxide |
| GPCRs | = | G-protein couple receptor |
| CREB | = | cyclic AMP response element binding protein |
| CSF | = | cerebrospinal fluid |
| $Cu^{+}$ | = | cuprous ion |
| $Cu^{2+}$ | = | cupric ion |
| $CuSO_4$ | = | copper sulfate |
| d | = | density |
| DMEM | = | dulbecco's modifide eagle's medium-lowglucose |
| EDTA | = | ethylenediamine-tetraacetic acid |
| EtOH | = | ethanol |
| fox-LDL | = | fully oxidized low density lipoprotein |
| FBS | = | fetal bovine serum |
| g | = | gram |
| HCl | = | hydrochloric acid |

## ABBREVIATIONS (CONT.)

| | | |
|---|---|---|
| HDL | = | high density lipoprotein |
| H | = | hour |
| IDL | = | intermediate low density lipoprotein |
| KBr | = | potassium bromine |
| KCl | = | potassium chloride |
| kDa | = | kilodalton |
| LDL | = | low density lipoprotein |
| LDLR | = | low density lipoprotein receptor |
| LGICs | = | Ligand-gated ion channel |
| LOX-1 | = | lectin-like oxidized-LDL receptor |
| LRP-1 | = | LDL receptor related protein-1 |
| mACh | = | muscarinic ACh receptors |
| MDA | = | malondialdehyde |
| $MgCl_2$ | = | magnesium chloride |
| M | = | molar |
| min | = | minute |
| mg | = | milligram |
| ml | = | milliliter |
| mM | = | millimolar |
| mox-LDL | = | mildly oxidized low density lipoprotein |
| µg | = | microgram |
| µl | = | microliter |
| µM | = | micromolar |
| $N_2$ | = | nitrogen gas |
| NaCl | = | sodium chloride |
| $Na_2EDTA$ | = | sodium ethylenediamine-tetraacetic acid |
| $Na_2CO_3$ | = | sodium bicarbonate |
| nAChRs | = | nicotinic ACh receptors |
| NTRs | = | neurotrophin receptor |
| Oxidized-LDL | = | oxidized low density lipoprotein |

## ABBREVIATIONS (CONT.)

| | | |
|---|---|---|
| OHC | = | hydroxycholesterol |
| pCREB | = | phosphorylated CREB |
| PBS | = | phosphate buffer saline |
| PVDF | = | polyvinylidene fluoride |
| ROS | = | reactive oxygen species |
| rpm | = | revolution per minute |
| SDS | = | sodiumdodecylsulfate |
| sAPP | = | soluble amyloid precursor protein |
| sAPP-α | = | soluble amyloid precursor protein alpha |
| sAPP-β | = | soluble amyloid precursor protein beta |
| TBARs | = | thiobarbituric acid reactive substances |
| TBS | = | tris buffer saline |
| TG | = | triglyceride |
| TLR-4 | = | toll-like receptor-4 |
| VGICs | = | Voltage gated ion channel |
| VLDL | = | very low density lipoprotein |
| VLDLR | = | very low density lipoprotein receptor |

# CHAPTER I

# INTRODUCTION

The first chapter contains four parts including the rational of the study, objectives, expected outputs, & expected outcomes of the study. The details of each part are described below.

**The rational of the study**

Alzheimer's disease (AD) is the most common cause of dementia in elderly people. The deposition of extracellular amyloid-β peptide (Aβ) and intracellular neurofibrillary tangle are the major pathology in AD [1]. Aβ induces pro-inflammatory responsive, mitochondria dysfunction and oxidative stress leading to neuronal cell death [1]. Accumulating evidence over the past two decades also suggests an association between cholesterol dyshomeostasis and the pathophysiology of sporadic AD [2]. As a model of hypercholesterolemia, ApoB-100 transgenic mice having high plasma lipid levels showed memory decline, increased cerebral Aβ accumulation and lipid peroxidation [3]. LDL receptor knockout mice demonstrated the increases in oxidative stress and mitochondrial dysfunction in cerebral brain [4]. In addition, cerebrospinal fluid (CSF) of AD patients showed the significant increase in IgG antibody to oxidized LDL [5] suggesting that oxidative stress and accompanying hypercholesterolemia may play a key role in AD progression.

It should be noted that cholesterol and lipoproteins in the circulation generally cannot pass into the brain because the blood brain barrier (BBB) functions to protect and limit the entry of substances into the brain. However, as diabetes and hypercholesterolemia models showed the increased BBB permeability and the leakage of microvascular IgG into the brain interstitium related to brain amyloid deposition and associated with sporadic AD [6, 7, 8]. Normally, IgG extravasation is not present in brain parenchyma, but these cholesterol-fed rabbits showed the increase of IgG extravasation in the brain [7]. ApoB is normally absent in brain [9] but it found in AD

[10] indicating that hypercholesterolemia can affect and disrupt the BBB integrity leading to BBB leakage.

Neurons express a number of receptors for cholesterol uptake such as low density lipoprotein receptor (LDLR), very low density lipoprotein receptor (VLDLR), LDL receptor-related protein-1 (LRP-1), & apoE receptor [11]. ApoB containing LDL can enter neurons via such receptors and promote amyloidogenic processing of APPβ by increasing activity and endolysosome accumulation of BACE-1, & the consequent increased Aβ formation [12]. Overexpression of human ApoB-100 increased the expression and processing of APP, promoted the formation of amyloid plaques, & induced extended neuronal death in transgenic mice [13, 14]. High cholesterol content in neuron of rabbit-fed high cholesterol diet was accompanied with accumulated Aβ, increased BACE activity and phosphorylated tau levels in hippocampus [15]. Thus, LDL cholesterol potentially was involved with many neurodegenerative processes of AD.

In addition to hypercholesterolemia, the role of cholesterol in AD has been controversial [16, 17], high total cholesterol levels and low high density lipoprotein (HDL) levels were found to increase risk of AD in general [17] and cholesterol level in AD brain are unchanged [18]. But key elements linking these AD risks are various oxidized cholesterol analogues (oxysterols). Brain cholesterol can be enzymatically and non-enzymatically converted into various oxysterol analogs [18]. 24S-hydroxycholesterol is a major form of oxysterol produced in brain and diffuses across BBB into the circulation. It regulates cholesterol synthesis, & a very potent inhibitor of Aβ production, but decreases in AD. 27-Hydroxycholesterol, which is produced peripherally, & it crosses the BBB, is cytotoxic, & enhances Aβ production in AD [18].

LDL is also susceptible to oxidative modification to form oxidized LDL in conditions of imbalance between free radical and detoxified by antioxidant enzymes [19, 20] and exposure to metal ion [21]. Antibodies against oxidized LDL were significantly increased in CSF of AD patients [5]. LDL protein carbonyl content and protein oxidation marker were found in AD plasma at higher levels than the control group [22]. In addition, oxidized LDL but not native LDL mediated cell apoptosis through elevating caspase-3 activity, expression of the receptor binding to oxidized

LDL (LOX-1), intracellular $Ca^{2+}$ and reactive oxygen species (ROS) [23, 24, 25]. Oxidized LDL also induced neurotoxicity by stimulated $Ca^{2+}$-dependent activation of mitogen-activated protein kinase (MAPK) signaling in culture primary striatal neuron [26]. In cell culture, LOX-1 has expressed in the cortex of rat and its expression has implication in neuronal apoptosis [27]. These data only suggest the neurotoxicity of oxidized LDL as neuron death. In addition, it might be other aspects of oxidized LDL involving in AD pathogenesis.

Therefore, this study has examined the hypothesis that the roles of LDL in various oxidation states in some clinically important pathology of AD. Two oxidized LDL preparations; mildly oxidized (mox-LDL) and fully oxidized-LDL (fox-LDL), were tested in comparison with native LDL on SH-SY5Y neuroblastoma cells by focusing on cellular dysfunctions linked to AD pathology. In the proposed study, we tested the effect of LDL and oxidized LDL on neurotoxicity and cellular oxidative stress, cellular acetylcholinesterase (AChE), amyloid precursor protein (APP) processing, & finally cyclic AMP response element binding protein (CREB) phosphorylation. The results from this study reveal the mechanism of LDL and its oxidized form on neurodegenerative process of AD development. This cellular experiment may be used as one of the cellular models for screening of various substances to prevent or treatment AD.

**Objectives**

1. To evaluate the neurotoxicity and cellular oxidative stress mediated by oxidized LDL
2. To investigate the type of cell death induced by oxidized LDL
3. To determine the effect of oxidized LDL on cellular dysfunction associated with AD pathogenesis including activity and expression of AChE, & APP processing
4. To investigate the effect of oxidized LDL on CREB signaling liked to neuronal plasticity involving cognitive functions

**Expected output of the study**

Expected output is to demonstrate the neurotoxicity of oxidized LDL. This study indicates that oxidized LDL can mediate various neurodegenerative associated with AD pathogenesis.

**Expected outcomes**

Oxidized LDL-induced neuronal damage can be further used as *in vitro* model for screening various substances to prevent or treat AD.

# CHAPTER II

# LITERATURE REVIEWS

## Alzheimer's disease (AD)

### 1. Pathology in AD

AD is the most frequent of neurodegenerative dementia in elderly people. Amyloid (Aβ) or senile plaques and neurofibrillary tangle are the pathology hallmarks of AD [28]. Brain of AD patients has the senile plaques and neurofibrillary tangle in the hippocampus, temporal cortex and nuclease basalis of Meynert (lateral septum) [28]. Aβ is a protein of 39-42 amino acids that is derived from the proteolytic of a large amyloid precursor protein (APP), a transmembrane protein. Soluble Aβ oligomers are aggregated to undergo fibrillogenesis and tendency to form plaques [29]. It induced pro-inflammatory response, mitochondria dysfunction, oxidative stress, & neuronal cell death, also decreases neurotransmitter such as acetylcholine that involves the formation of learning and memory [1]. Hyperphosphorylation of tau protein involves the formation of neurofibrilary tangles that is associated with AD development [28]. Tau is a microtubule associated protein located in neuronal cytoplasm. The function of this protein is to assemble and stabilize the microtubules to convey cell organelles, glycoprotein, & other important materials throughout the neuron. The ability of its function is associated by phosphate group.

### 2. Clinical features

AD brains have specific regions of neuronal degeneration which is associated with clinical symptoms. The deposition of Aβ begins in the hippocampus and spread to the CA1 area of the hippocampus and the cholinergic nuclei and then progressing to the frontal regions of the neocortex and finally spreading to the sensory and motor regions [30]. The progression of phosphorylated tau is also the similarity pattern with Aβ progression [30].

An early clinical symptom is difficulty to remember names and recent event. Apathy and depression are also often in early symptoms. Later symptoms include impaired judgement, disorientation, confusion, behavioral changes, &

difficulty to speak, swallow, & walk [28]. The physical and neurological symptoms of AD do not affect motor systems until in late state of the disease.

Neuroimaging including computed tomography (CT) and magnetic resonance imaging (MRI) have been used to identify AD and other dementias. AD brain shows hippocampal atrophy and cortical atrophy [28]. The imaging biomarker including single photon emission tomography (SPECT) and positron emission tomography (PET) have been used to diagnose AD and reveal the hypoperfusion or hypometabolism in the posterior temporal-parietal cortex of AD [28].

**3. Type of AD**

Base on the onset periods of the disease, AD have been generally clarified into two types including familial AD (FAD) and sporadic AD [31, 32]. FAD is known as early-onset AD (EOAD) and appears prior to 65 years of age [2, 31]. FAD is found less than 5% of all AD cases. It is inherited with the mutation of the gene which encoded the proteins involving in the Aβ production including APP gene on chromosome 21, presenilin 1 on chromosome 14 and presenilin 2 on chromosome 1 [31]. The common type of AD is sporadic AD which is known as late-onset AD (LOAD) and normally diagnosed over 65 years of age [31]. Sporadic AD is estimated more than 95 % of cases [31]. The apolipoprotein E (*APOE*) gene on chromosome 19 especially, apoE4 is closely associated with the increased risk in sporadic AD [31, 32]. The mutation of other genes may also relate to AD such as clusterin (*ApoJ* on chromosome 8), phosphatidylinositol-binding clathrin assembly protein (*PICALM* on chromosome 11), complement receptor 1 (*CR1* on chromosome 1), bridging integrator 1 (*BIN 1* on chromosome 2) and sortilin-related receptor 1(*SORL1* on chromosome 1) [2, 32].

**4. Risk factor of AD**

AD has multiple risk factors including age, genetic and brain injury. Other risk factors have been associated with AD such as elevated serum cholesterol, diabetes, hypertension, smoking and obesity [31, 32]. The cerebrovascular disease or brain infarction is also considered as the risk factor of the development AD and closely involved in vascular dementia [32]. Moreover, hormones such as estrogen and oxidative stress have been reported to associate with the development of AD pathogenesis [1, 33].

**5. Drug used in AD**

**Cholinesterase inhibitors (AChEIs)**

The AChEIs used to inhibit both acetylcholinesterase and butyrylcholinesterase responsible for the breakdown of acetylcholine hence increasing its availability at the synaptic cleft and boost cholinergic neurotransmission in forebrain regions, & this is thought to contribute clinical benefits of AD [34, 35]. Three AChEIs have been used to treat AD patient including donepezil, rivastigmine and galantamine. AChEIs are approved for mild to moderate AD excepted donepezil that is also approved for severe AD [34, 35]. Donepezil is a selective reversible inhibitor of acetylcholinesterase. Rivastigmine is an inhibitor of both acetylcholinesterase and butylcholinesterase. Galantamine can stimulate nicotinic acetylcholine receptors, in addition to inhibition of cholinesterase activity.

**N-methyl-D-aspartic acid (NMDA) glutamate receptor antagonists**

The enhancement of the excitatory effects of the neurotransmitter glutamate may play a role in the pathogenesis of AD [36]. The current drugs used to treatment of AD patient such as memantine. Memantine is a N-methyl-D-aspartate (NMDA) non-competitive glutamate receptor antagonist. It is thought to protect neurons from excessive glutamate activity that results in excitotoxicity [36, 37]. Other properties of memantine that could also be relevant in AD including its ability to enhance long-term potentiation [38] and decreased tau hyperphosphorylation [36, 39].

**Cellular mechanism in AD**

**1. Role of APP metabolism and Aβ in AD**

APP is a large 110-120 kDa type I transmembrane glycoprotein that contains Aβ peptide which is found in the AD amyloid plaque [40, 41]. It is generally located within plasma membrane and consisted of a large N-terminal domain outside the cell and a short C-terminal domain inside the cell [41]. APP contains 695-770 amino acids [41]. Three isoforms found in the brain are the APP695, APP751 and APP770 [42, 43, 44]. The APP695 is the shortest isoform but it is expressed at higher level compared to APP751/770 [45]. Moreover, APP has been found in the membrane of the trans-Golgi network (TGN), endoplasmic reticulum (ER), endolysosomes and mitochondria [46]. Functions of APP are involved in the growing of cells, helping

neuron differentiation, cell adhesion, calcium metabolism, synapses and protein trafficking [41, 42, 45].

### 1.1 APP processing

APP can be processed via non-amyloidogenic and amyloidogenic pathways which are sequential steps involving three enzymes activities including α, β, & γ seretase [42, 45].

**Non-amyloidogenic pathway**

Alpha -secretase is the first enzyme to process APP within the Aβ domain after lysine residue 16 on the cell surface and release the extracellular secreted APP α (sAPPα) fragment and C-terminal fragment (CTF-83) of APP 9 (Figure 1) [45]. The CTF-83 is then cleaved by γ-secretase and generates the P3 peptide and the APP intracellular domain (AICD) [45]. The sAPPα have many physiological functions such as protection the neuron, regulation of the stem cell production, promotion the formation of synapse, cell adhesion, & brain development [47]. The p3 peptide is quickly undergo degradation after cleavage and has no important function whereas AICD have showed to regulate transcription and intracellular trafficking [45, 47].

**Amyloidogenic pathway**

Beta-site APP cleaving enzyme 1 (BACE 1) cleaves the full-length APP at the beginning of a sequence of Aβ and releases the extracellular secreted APP β (sAPPβ) fragment and C-terminal fragment (CTF-99 or CTF-89 (Figure 1) [45]. The CTF-99 or CTF-89 are then cleaved by γ-secretase to produce Aβ protein and AICD [45]. The cleaving site of γ-secretase within transmembrane domain of APP can produce various type of Aβ such as Aβ40 or Aβ42 [42, 45]. Then, Aβ is secreted into extracellular by exocytosis. The sAPPβ has been reported to act as ligand for a Death Receptor 6 and cell death [45, 47].

### 1.2 Aβ in AD

Aβ is a protein component of amyloid plaque in AD brain [41]. It is released from APP metabolism. The concentration of Aβ is about 221 μg/g of brain tissue in healthy people brain but in the brain of AD patient is 406 mg/g of brain tissue [48]. Aβ is believed to play some roles in cell survival, cell excitability, immunity, learning and memory [41]. Aβ can be found in both extracellular and intracellular in AD [49]. It is commonly divided to two types including Aβ40 and Aβ42 [29]. Aβ40 is

usually found in healthy people more than Aβ42 but Aβ42 has more toxicity than Aβ40 [29]. Aβ42 can be aggregated together leading to Aβ deposition and later fibrillogenesis [29]. Since the end of Aβ42 peptides contain two water repelling amino acids [29] therefore it can attract and form plaque in water based fluids [29]. Thus, the increased Aβ42 level is typical to develop AD [29].

Aβ is degraded by several enzymes within the cells including insulin degrading enzyme (IDE), neprilysin, plasmin, cathepsin, endothelin converting enzyme and the matrix-metalloproteinase family [41, 46]. A study showed that these enzymes are decreased in AD [46]. In the peripheral system, Aβ is eliminated by liver and kidney [41].

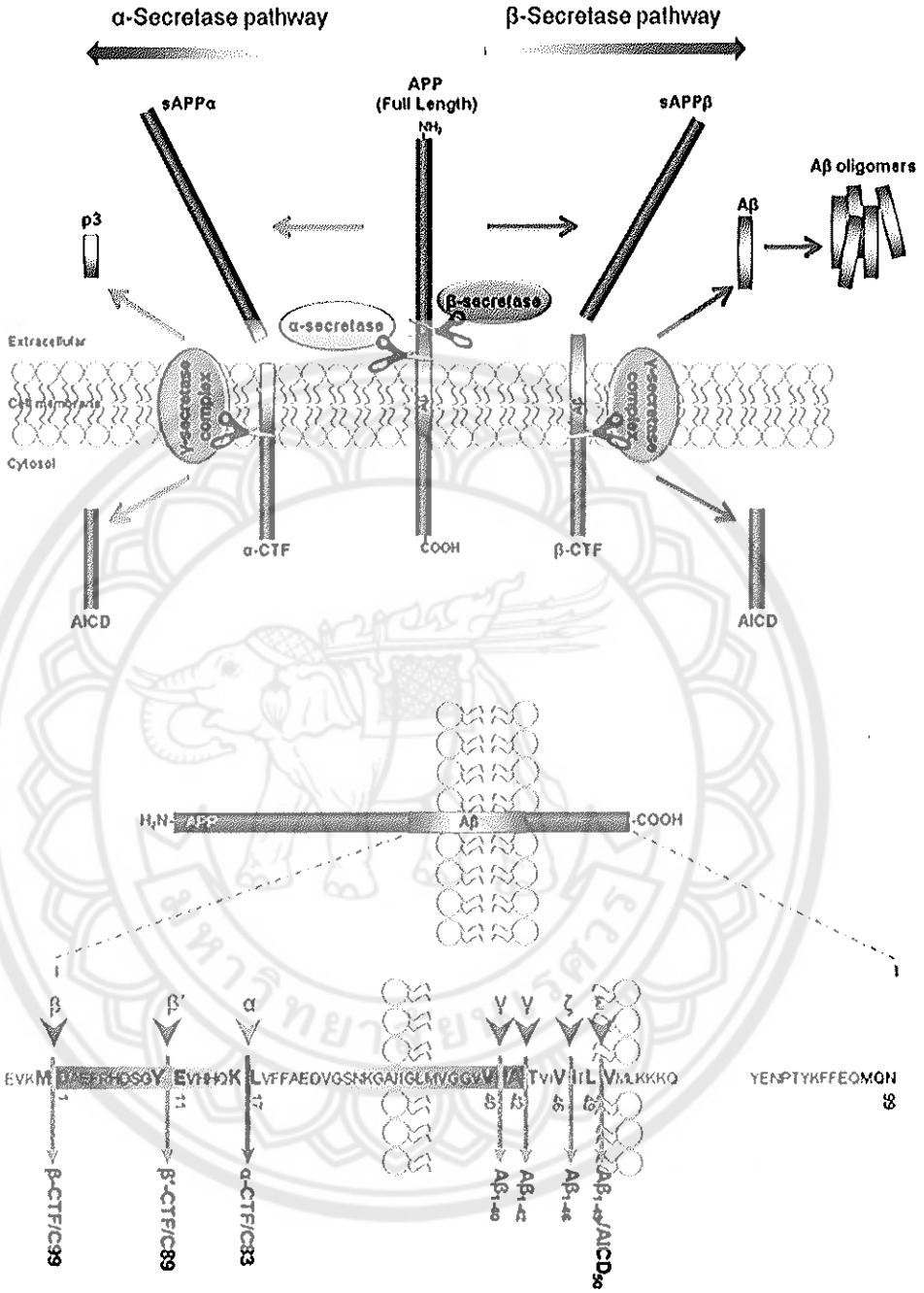



**Figure 1 Schematic of the processing of APP using β, α, & γ secretase** [2]

## 2. Role of cholinergic system in AD

### 2.1 Cholinergic system

Acetylcholine (ACh) is a neurotransmitter in the cholinergic neuron. It is released into the synaptic cleft by exocytosis and hydrolyzed by acetylcholinesterase (AChE) enzyme (Figure 2) [50]. The cholinergic neuron is first located in the telencephalon, medial and ventral to the basal ganglia [50]. It includes the basal nucleus of Meynert, which provides cholinergic innervation to the entire neocortex, amygdala, hippocampus, & thalamus. The medial septal nuclei provide cholinergic innervation to the cerebral cortex, hippocampus, & amygdala (Figure 3) [50]. The second constellation includes cholinergic neurons located in the dorsolateral tegmentum of the pons that project to the basal ganglia, thalamus, hypothalamus, medullary reticular formation, & deep cerebellar nuclei (Figure 3) [50].

There are two forms of AChE in the CNS. A tailed form of AChE (AChE-T) is referred to the AChE C-terminal sequence [51]. AChE-T is transcripted and translated from exon 6 of the AChE gene [51]. It mainly occurs as a tetramer and anchored on cellular membrane via the Proline Rich Membrane Achor. AChE-T is the most common form in the CNS. Another form of AChE is a readthrough AChE (AChE-R) which is mainly monomeric and soluble splice as variant of AChE. It is transiently expressed and being implicated in stress related process [51].

### 2.2 Cholinergic system and AD

The cholinergic activity is closely associated in learning and memory processing. Cortex and hippocampal of AD patients exhibited atrophy and loss of cholinergic neurons [50]. In severe stage of AD, patients exhibited significantly increased acetylcholinesterase (AChE) activity [52]. AChE presents around the AD brain may enhance amyloid fibrillogenesis and amyloid toxicity [53, 54]. Thus, the initial attempts to treat AD is to increase the cholinergic function by administration of acetylcholinesterase inhibitor [35].

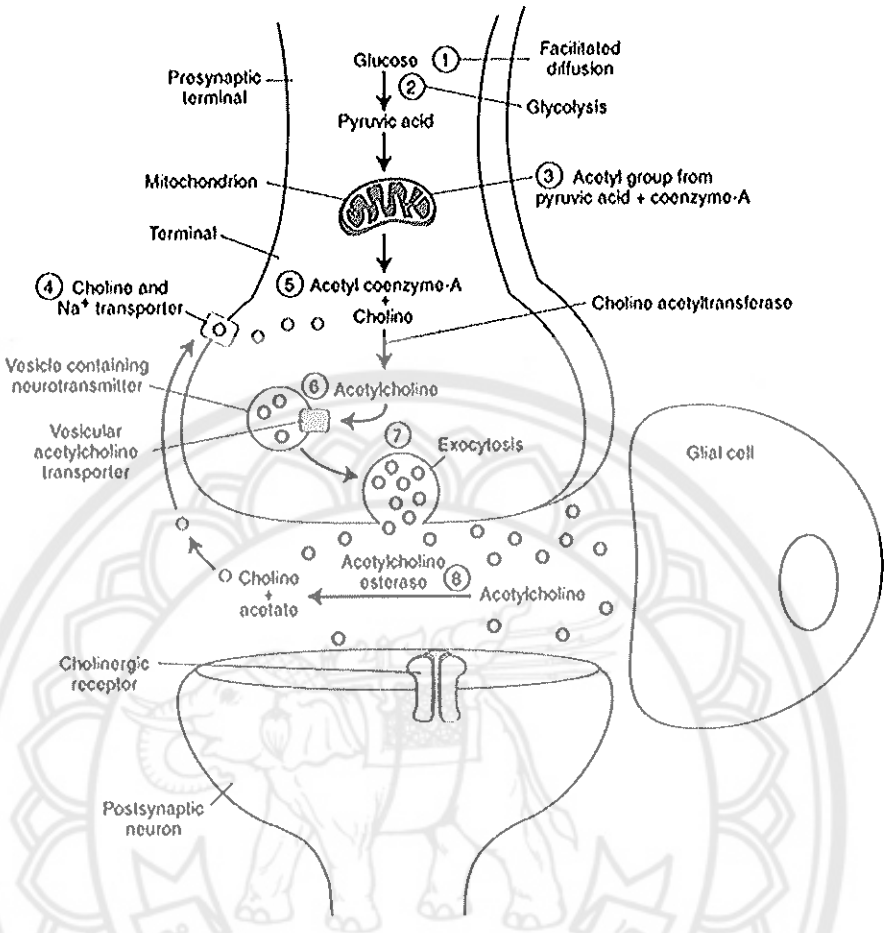



**Figure 2 The synthesis and release of acetylcholine** [50]

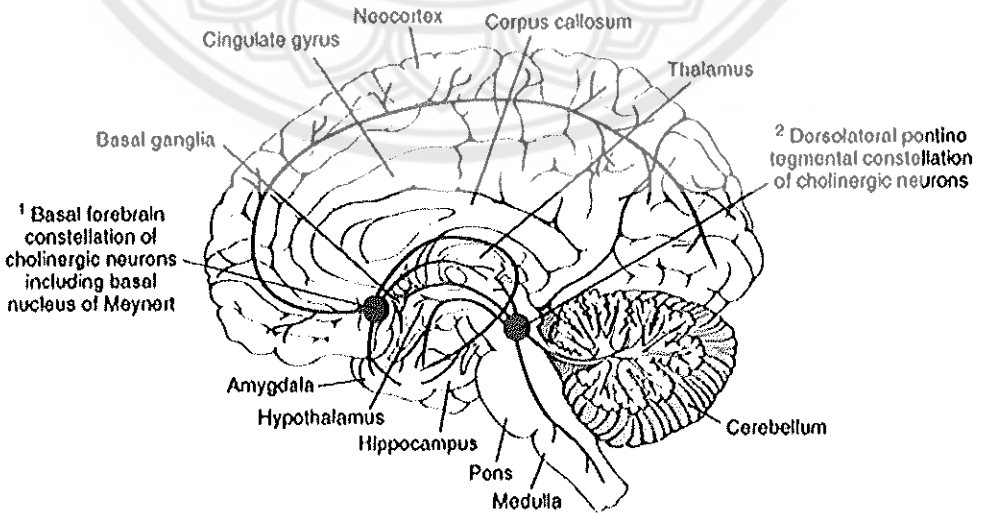



**Figure 3 The cholinergic pathway and projection area** [50]

## 3. Cell death in AD

### 3.1 Type of cell death

The imbalance between the cell division and the cell death is leading to a variety of disorder. When rate of cell division is higher than rate of cell death, this causes hyperplasia, cancer and autoimmune disease [55, 56]. If the rate of cell death is higher than the rate of cell division, it can cause degenerative diseases and ischemic injury [56]. There are two types of cell death including apoptosis and necrosis [57].

Apoptosis is a process of program of cell death that occurs during development. There are two main signaling pathways to activate the apoptosis. The first is extrinsic pathway or death receptor pathway involving the activation of the death receptor at cell surface such as the tumor necrosis factor receptor family (TNF-R) [57]. It is activated by binding to their ligand and initiate the caspase 8 [57]. The second is the mitochondria pathway or intrinsic pathway [55]. It is activated by internal factor such as DNA damage. After DNA damaged, the p-53 protein is released to activate the cytochrome C release from the mitochondria [55]. A variety of stresses can activate the mitochondria and induce the leakage cytochrome C and apoptosis [57]. After cytochrome C release, it can bind to Apaf-1 to form apoptosome in the present of ATP and subsequently activates caspase 9 [57]. Both pathways converge on the effector as caspase 3 and caspase 7 and lead to apoptosis [56]. There are many factors having ability to stimulate apoptosis such as DNA damage, oxidative stress, toxin, certain drug and hormones [56].

Necrosis is called accidental cell death. It occurs when cells are exposed to physical or chemical environments such as hyperthermia, hypoxia, radiation, low pH, & cell trauma which causes cell injury and plasma membrane distruption [56]. The features of necrosis is characterized by cell swelling and lysis [56]. Cell swelling causes intracellular organelles such as mitochondria, rER and nucleus dysfunction [56]. After cell lysis, cytoplasm contents such as lysosomal enzymes are released into extracellular space [56]. The differences between cell necrosis and apoptosis are shown in Table 1.

### 3.2 Cell death and AD

Apoptotic and necrotic deaths have been found in AD brain tissue. Both apoptotic and necrotic pathways can be mimicked by employing a variety of

models systems of AD-associated nerve cell degeneration [58]. The expression of caspase-3 expression is high and shows colocalization with the neurofibrillary tangles and senile plaques in AD brain [59]. Caspase-3 has been associated with the proteolytic of APP and enhance the Aβ production [58]. Aβ mediated apoptosis in cortical neuron through the activation caspase-3 and also induced the loss of membrane integrity detected by the release of LDH [60]. Thus apoptosis and necrosis might be overlapping in the consequence of AD pathogenesis.

**Table 1 The characteristic and differences between apoptosis and necrosis** [58]

| Apoptosis | Necrosis |
|---|---|
| Cell shrinkage | Cell swelling |
| Membrane blebbing | Disintegration of membranes |
| Chromatin aggregation (DNA-laddering in agarose gel) | Random degeneration of DNA (DNA-smear in agarose gel) |
| Formation of apoptotic bodies | Cell lysis |
| Organelles and membranes remain intact | Disintegration of organelles |
| Enzymatic process/caspase-activation | Disturbed ion homeostasis |
| Energy dependent | Energy independent |
| Well-control cell death | Insult-induced spontaneous cell death |
| No inflammatory response | Inflammation |

## 4. Role of CREB signaling in AD

### 4.1 Physiology of CREB functions in neuron

The biochemical signaling for memory formation and cognitive function in neuron have been studied and found that it was involved cyclic AMP respone element binding protein (CREB) function [61]. CREB, a nuclear transcription factor and is 43 kDa [61]. CREB is phosphorylation and bind to the DNA cAMP response element (CRE) within the nuclear. It is phosphorylated at Ser-133 by several kinases such as $Ca^{2+}$/calmodulin-dependtdent kinases (CaMKs), mitogen activated protein kinases (MAPK), protein kinases C (PKC) and MAPK activated ribosomal S6 kinases (Figure 4) [61, 62, 63].

CREB is believed to be responsible for transcriptional activation leading to gene products such as brain derived neurotrophic factor (BDNF) that plays a key role in synaptic plasticity required for long-term memory in response to environmental learning [61]. Moreover, the activation on CREB can produce other gene products that involved in neuronal cell function and survival such as neurotrophin, tyrosine hydroxylase and neuropeptides [62].

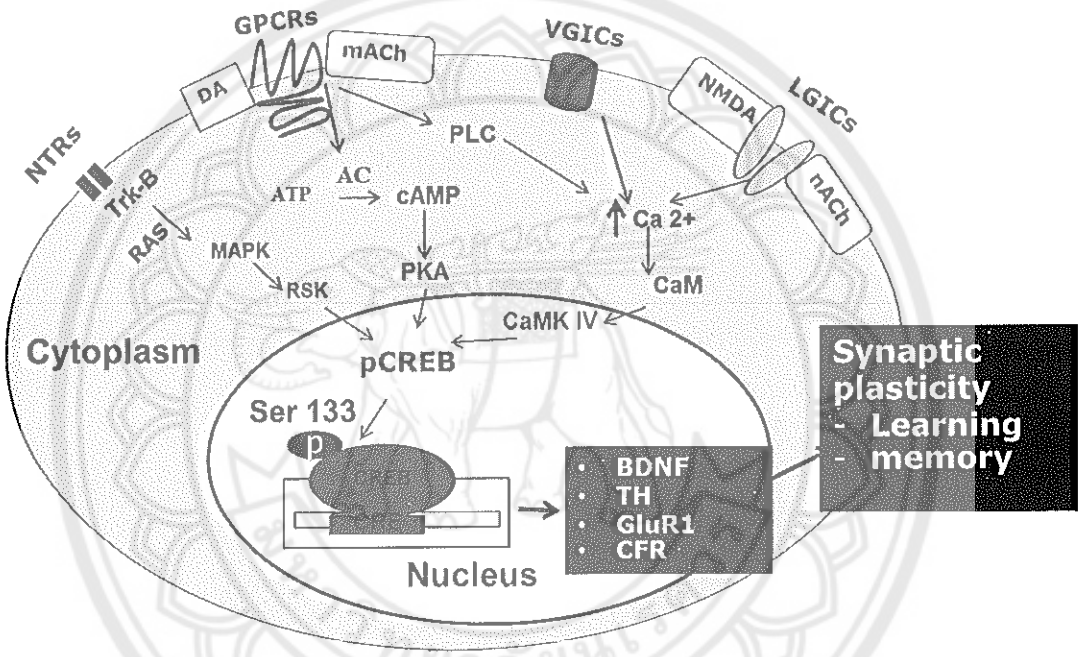



**Figure 4 Schematic of CREB phosphorylation** [61]

**Note:** NTRs = neurotrophin receptor such as tyrosine kinase receptor (TrkB); GPCRs = G-protein couple receptor such as dopamine and mACh receptor; VGICs = Voltage gated ion channel; LGICs = Ligand-gated ion channel such as NMDA and nACh receptor; CaMK = $Ca^{2+}$/ Calmodulin-dependent protein kinase; CREB = Cyclic AMP response element binding protein

### 4.2 CREB and AD pathology

The impaired CREB phosphorylation has been reported to involve with various pathogenesis of neurodegenerative disorder in particular AD [61]. Aβ was reported to interfere the signaling pathway involving in cognitive function. Aβ decreased CREB phosphorylation via the pathway of NMDA stimulation in cultured cortical neuron and resulted in reduction of BDNF gene expression [64]. Aβ inhibited long-term potentiation by decreasing cAMP and PKA-cascade CREB phosphorylation [65]. The impaired CREB phosphorylation in this experiment can be rescued by phosphatase inhibitor rolipram [65]. Transgenic mice overexpressed Aβ exhibited reduction of phosphorylation of ERK and CREB in cortex compared with wild type [66]. In addition, the decrease in phosphorylated CREB has been reported in postmortem brain of AD patients [67]. Aβ-induced oxidative stress also showed a decrease in CREB level in hippocampus and cortex of mouse brain [68]. In cerebellar granule neuron cells, oxidative stress decreased pCREB within 1 h treatment, whereas the loss of total CREB protein was observed 2 h after $H_2O_2$ treatment [69]. Taken all data together, CREB signaling has close relationships with AD.

## 5. Role of oxidative stress in AD

### 5.1 Oxidative stress

Oxidative stress is the condition of imbalance between the production of oxidants species and antioxidant system. It can damage cells, tissues and organs in various system [1]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are types of oxidants that found in biological system [70]. ROS includes superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide ($H_2O_2$), & hydroxyl radical ($OH^{\cdot}$) [70]. Mitochondria is major organelle in the cellular respiratory that produces the ROS [71]. Oxidants can damage the macromolecules including protein, lipid, & DNA [70]. The nitrosative stress was induced by RNS. Nitric oxide is synthesized via the activity of nitric oxide synthase- (NOS) [70]. Both ROS and RNS can induce cell death. Mildly oxidative stress can trigger cell apoptosis but the highly oxidative products induce cell necrosis [72].

The antioxidants system can detoxify the oxidants in biology system [70]. Antioxidant can prevent cell damaging from oxidant species or scavenge the formation of radical species. There are several antioxidant in cell biological system

such as superoxide dismutase (SOD), glutathione peroxidase, & catalase. SOD catalyzed the superoxide to $H_2O_2$ and $H_2O$ [70]. Catalase catalyzed $H_2O_2$ to $H_2O$ and $O_2$ [70]. Glutathione peroxidase detoxified $H_2O_2$ to $H_2O$ and GSSH [70].

**5.2 Oxidative stress and AD**

Oxidative stress has been reported as an early event in the AD brain and might play an important role in the development of AD. The metal ions such as Fe, Al and Hg are higher level in AD brain and are capable to stimulate the free radical generation [73]. Oxidative stress can damage the macromolecules in cells, tissues and organs and might be important in the pathogenesis of neuronal degeneration [1]. AD brain have been found increased intermediates compounds of oxidative stress events including increased the lipid peroxidation; decreased polyunsaturated fatty acids; increased protein and DNA oxidation; diminished energy metabolism; and decreased cytochrome c oxidase [73]. Advanced glycation end products (AGE), malondialdehyde, carbonyls, peroxynitrite, heme oxygenase-1 and SOD-1 are represented in neurofibrillary tangles and AGE, heme oxygenase-1, SOD-1 are also found in senile plaques [73]. Aβ is also capable to generate the free radicals and induced neuronal cell death [1]. From these mentioned studies free radicals are possibly involved in the pathogenesis of neuron death in AD.

**Hypercholesterolemia in AD**

**1. Cholesterol metabolism in the circulation**

The major lipids in the plasma are cholesterol (free and esterified cholesterol), fatty acids, triglycerides (TG) and phospholipids (PL) [74]. They play important role as components in cell membrane that regulate membrane fluidity (cholesterol, phospholipid), precursor of steroid hormones and bile salt (cholesterol) and energy metabolism (TG and fatty acids) [75]. Lipids are packed and transported in the plasma in association with protein as lipoproteins [74]. Lipoproteins are composed of a hydrophobic core of TG and cholesterol esters (CE) and surrounded by a hydrophilic surface of free cholesterol (FC), phospholipid and apolipoproteins [74]. Plasma lipoproteins can be typically separated to five subclasses by the basis of their densities including chylomicrons (CM), very low density lipoproteins (VLDL),

intermediated-density lipoproteins (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) (Table 2) [76].

LDL is the main carriers of cholesterol in the circulation and has a key role in cholesterol transfer and metabolism [74]. LDL is generated from the metabolism of VLDL (triglyceride-rich lipoproteins) [75]. In the VLDL-IDL-LDL cascade, the action of lipoprotein lipase and hepatic lipase depleted TG and apolipoprotein except the apoliporotein B-100 (apoB-100) remains in the particles [75]. LDL can also be synthesized directly by the liver [74]. LDL can bind to the LDL receptor on cell membrane that recognizes apoB-100 and passes through the endothelial cells [77]. Then, LDL is taken up and undergo by lysosome degradation followed by the release of free cholesterol into the cytosol [75]. The classical LDL receptors such as scavenger receptor also recognize chemically and biologically modified lipoprotein including oxidized LDL[78].

**Table 2 Composition of lipid, apolipoprotein of human plasma lipoproteins** [76]

| Parameter | Chylomicron | VLDL | IDL | LDL | HDL |
|---|---|---|---|---|---|
| Hydrated density (g/ml) | 0.93 | 0.97 | 1.003 | 1.034 | 1.121 |
| Solvent density for isolation (g/ml) | < 1.006 | < 1.006 | 1.006-1.019 | 1.019-1.063 | 1.063-1.210 |
| Molecular weight | $(0.3\text{-}30)\times10^9$ | $(5\text{-}10)\times10^6$ | $(3.9\text{-}4.8)\times10^6$ | $2.75\times10^6$ | $(3.6\text{-}17.5)\times10^5$ |
| Diameter (nm) | >70.0 | 25.0 – 70.0 | 22.0 – 24.0 | 19.6 – 22.7 | 4 – 10 |
| Electrophoretic mobility (paper, agarose) | Origin | Pre-β | Broad β (between β and Pre-β | β | α |

**Table 2 (cont.)**

| Parameter | Chylomicron | VLDL | IDL | LDL | HDL |
|---|---|---|---|---|---|
| Composition (% by weight) | | | | | |
| FC | 2 | 7 | 8 | 8 | 6* |
| CE | 5 | 12 | 22 | 37 | 13* |
| PL | 7 | 18 | 25 | 22 | 27* |
| TG | 84 | 51 | 30 | 10 | 3* |
| Protein | 2 | 10 | 15 | 22 | 50* |
| Apoproteins (% total apolipoprotein) | | | | | |
| AI | 7.4 | Trace | - | - | 67 |
| AII | 4.2 | Trace | - | - | 22 |
| B-100 | Trace | 36.9 | 50-70 | 98 | Trace |
| B-48 | 22.5 | Trace | Trace | - | - |
| CI, CII, CIII | 66 | 49.9 | 5-10 | Trace | 5-11 |
| EII, EIII,EIV | - | 13.0 | 10-20 | Trace | 1-2 |
| D | - | - | - | - | Trace |
| Synthesis | Intestine | Liver, Intestine | Lipolysis of VLDL | Lipolysis of VLDL, via IDL | Liver, Intestine; lipolysis of CM & VLDL |

**Source:** * From Jackson RR, Morrisett JD, Gotto AM. Lipoproteins and lipid transport: Structural and functional concepts. In: hyperlidemia: Diagnosis and therapy. Rifkind BM, Levy RI, Eds. New York, Grune & Stratton, 1977:1-16)

**Note:** FC= Free cholesterol, CE= Cholesterol ester, PL= Phospholipid, TG= Triglyceride

**2. Cholesterol metabolism in the brain**

During brain development, neurons can synthesize cholesterol using for growth and synaptogenesis processes [2]. On the other hand, mature neurons receive cholesterol from astrocytes [2]. Astrocytes can synthesize cholesterol using acetyl-CoA and hydroxymethylglutarly-CoA (HMG-CoA) reductase enzyme and then formed ApoE-cholesterol complex [2]. ApoE-cholestrol complex is transported to neuron by endocytosis via various LDL receptor families such as LDL receptor-related protein-1(LRP-1), LRP and ApoE type-2 receptor (ApoER2) [2, 79]. ApoE-cholesterol complexes are delivered to endolysosome and the cholesterol ester is hydrolyzed to free cholesterol within lipoprotein complexes [2]. This free sterol is exported out of endolysosome by Niemann-Pick C protein [2]. Free cholesterol is also removed from neuron by conversion to 24S-hydroxycholesterol (24-OHC), an oxidized lipophilic metabolite that can cross the BBB to the circulation [2, 80]. Inversely, peripheral cholesterol can pass BBB into the brain as 27-hydroxycholestreol (27-OHC) metabolite [80, 81]. These metabolites can conveniently pass between the brain and the circulation by concentration gradient (Figure 5) [81].

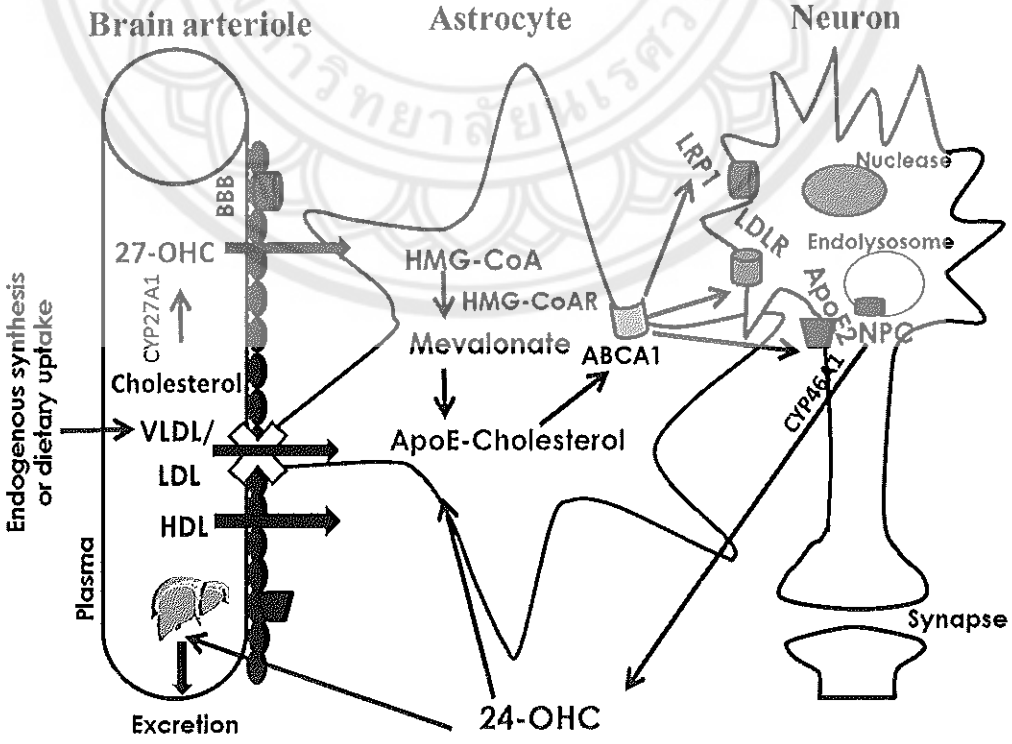



**Figure 5 Schematic of brain cholesterol metabolism and oxysterol** [2, 82]

**3. Lipoproteins in CNS**

In addition to the plasma, lipoprotein are found in other body fluids such as the cerebrospinal fluid (CSF) [83]. The choroid plexus produced CSF lipoprotein because the plasma lipoproteins cannot pass to BBB [83]. Size of CSF lipoprotein is 7-15 nm and their density is closed to plasma HDL. They contain the core lipid CE and the main apolipoprotein is apoE [79, 84, 85]. In the CNS, apoE is also presented in the astrocytes and microglia [83]. The astrocytes can synthesize the lipoproteins and carry the cholesterol to the mature neuron [9].

**4. The LDL receptor family member in AD**

There are various member of LDL receptors. The LDL receptor includes the LDL, LDL-related protein (LRP1 or LRP), LRP1B, megalin/LRP2, the very low-density lipoprotein receptor (VLDLR), apoE receptor 2 (apoER2), LRP4/MEGF7, LRP5, LRP6 and sorting protein-related receptor containing LDLR class A repeats (sorLA) or LR11 [86]. They share several structure motifs and functional characteristics including ligand-binding complement type repeats, epidermal growth factor (EGF) receptor like repeats, YWTD β-propeller domain that is single transmembrane domain, binding of apolipoprotein E (apoE), a protein involved in cholesterol transporter, & endocytic motif within their cytoplasm domains [86]. The LDL receptor family have various function from cholesterol metabolism to cell signaling [86].

There are at least four LDLR families such as LRP1, LRP1B, SorLA/LR11 and apoER2 that interact with APP and regulate APP trafficking [86]. The LRP1 promotes APP endocytosis and processing to form Aβ [86]. LRP 1 binds to several ligands that are associated with to AD such as APP and Aβ in addition to apoE [86]. From *in vitro,* it mediates the clearance of Aβ ether by biding with to Aβ itself or Aβ complexed to this ligands [86, 87]. Moreover, LRP1 and its ligand are found together with amyloid plaque in AD brain [88]. SorLA/LR11 also regulates the APP trafficking and reduces APP processing to form Aβ [86]. ApoER2 reduces APP endocytosis and is able to promote or inhibit APP processing [86].

**5. Hypercholesterolemia and AD**

Hypercholesterolemia is a well-known risk factor for cardiovascular disease while its association with AD progression are controversy [89]. Overexpression

of human ApoB-100 increased the expression and processing of APP, promoted the formation of amyloid plaques, & induced extended neuronal death in transgenic mice [13, 14]. High cholesterol content in neuron of rabbit-fed high cholesterol diet was accompanied with accumulated Aβ, increased BACE activity and phosphorylated tau levels in hippocampus [15]. Lipoproteins are not able to cross the BBB. However, a damaged or dysfunctional cerebrovasculature under a hypercholesterolemic condition can cause extravasation of serum components into and through the walls of cerebral small vessels. Rabbit fed cholesterol enriched diets have been used as a model of neurovascular disorders associated with sporadic AD [6, 7]. IgG extravasation is normally not present in brain parenchyma, but the increased immunoreactivity of IgG extravasation was found in the brain of cholesterol-fed rabbits, indicating that hypercholesterolemia affected and disrupted the BBB integrity and leading to BBB leakage [6, 7]. Moreover, in the brain of AD patients has been found ApoB immunoreactivity in senile plaque and vascular amyloid and neurofibrillary tangles [10]. The disruption of microvascular endothelial cells and BBB integrity is thought to be associated in hypercholesterolemia and AD development.

**Oxidation of cholesterol and lipoprotein in AD**

**1. Oxidation of Cholesterol**

The intermediates of cholesterol oxidation in PNS are oxidized cholesterol or oxysterol such as 6-cholesten-5α-hydroperoxide, 7-oxoholesterol (7-ketocholesterol), 7-OHC (7*β*-hydroxycholesterol), 7-dehydrocholesterol, 25-OHC and 27-hydroxycholesterol (27-OHC) [82]. 27-OHC is oxidized by enzymatic pathway in PNS that can pass directly through BBB into the brain [82]. Brain cholesterol is oxidized to form 24-hydroxycholesterol (24-OHC) by enzyme CYP46A1 and secreted out into PNS to be metabolited in the liver [82]. 24S-hydroxycholesterol is a major form of oxysterol produced in brain and diffuses across BBB into the circulation. It regulates cholesterol synthesis, & a very potent inhibitor of Aβ production, but decreases in AD [18]. 27-Hydroxycholesterol is produced peripherally, & it crosses the BBB, is cytotoxic, & enhances Aβ production in AD [18].These evidence might be important key link between oxidized cholesterol and AD progression.

**2. Oxidation of lipoprotein**

Lipoprotein oxidation is generated in the subendothelial space of the arterial wall or intima and then oxidation-LDL returns to the plasma compartment [90]. The artery wall consist the cells that secrete oxidative substance that further initiate modification of LDL, such as macrophage, endothelial cells and smooth muscle cells [91, 92]. Oxidative modification of LDL particles are well known association with the pathogenesis of atherosclerosis.

For in vitro experiment, many agents can be used to induce the lipid oxidation including copper or iron ions, ceruloplasmin, 15-lipoxygenase, peroxynitrite, the free radical generates 2,2'-azobis(2-aminopropane) dihydrochloride (AAPH) and 2,2'-azobis(2,4-dimethyl-varelonitrile) (AMVN) [20, 21]. The most common method for the initiation of LDL oxidation is using copper ion [21]. Copper ions participate in redox cycling reactions [21]. Protein component of LDL such as ApoB is required for metal-induced peroxidation [21]. Copper ion can bind to the various groups of amino acid on ApoB such as histidine and cysteine [21] and then react with the endogenous lipid hydroperoxides (LOOH) [21]. The peroxyl (LOO•) and alkoxyl (LO•) radicals generated in the above reaction are believed to initiate oxidation of the polyunsaturated fatty acid through hydrogen-atom capture [21]. $Cu^{2+}$-induced LDL oxidation results in the formation of reactive aldehydes such as malondialdehyde and 4-hydroxynoneal [21]. Oxidized LDL consists of a number of oxidation products shown as Table 3.

**Table 3 List of lipid/protein oxidation products generated during the oxidation of LDL** [93]

| Macromolecules | Lipid/protein oxidation products |
|---|---|
| Fatty acid | - Free and esterified fatty acid peroxides, such as 13-hydroperoxylinoleic acid (13-HPODE)<br>- Free and esterified fatty acid hydroxides, such as 13-hydroxylinoleic acid (13-HODE)<br>- Prostaglandin-like products, such as isoprostans in free and esterified form<br>- Aldehydes such as malondialdehyde (MDA), 4-hydroxynonenal, & hexanal<br>- Core aldehydes that contain esterified lipid backbone, such as oxovaleryl, phosphatidylcholine<br>- Pentane and other hydrocarbons |
| Lipid derived products | - Lysophosphatidylcholine<br>- Cholesterol oxidation products, such as 7-keto cholesterol<br>- Internally, modifiled phosphatidyl ethanolamine/serine products |
| Protein oxidation products | - Protein carbonyls<br>- Non-enzymatic proteolyzed fragments<br>- Modified cysteine, cysteine, histidine, methionine, lysine, arginine, tryptophan, & tyrosine<br>- Protein cross-link due to tyrosine cross-line as well as due to bifunctional aldehydes<br>- Lipid-protein adducts which could be classified as ceroids (lipofusion)<br>- Many of the above changes as well as conformation changes might lead to antigenicity |
| Other changes | - Increased buoyant density<br>- Increased negative charge<br>- Loss of characteristic yellow color (human)<br>- Loss of enzyme activities associated with LDL |

**2. Oxidized Low density lipoprotein receptor**

Scavenger receptors including CD36, scavenger receptor class A (SR-A), scavenger receptor-BI (SR-BI) and lectin-like oxidized LDL receptor-1 (LOX-1) are cell surface proteins that bind to various native and modified substances and subsequently mediate cells adhesion, endocytosis and activation of intracellular signaling [94]. The main receptor of oxidized LDL is LOX-1 [94].

LOX-1 is a scavenger receptor of a 50 kDa type 2 membrane proteins [94]. It mediates the binding, internalization and degradation of oxidized LDL in endothelial cells [95]. However, it is also expressed in other cell types such as rodent dorsal root ganglion (DRG) [24, 96], mouse hippocampal neurons (HN33) [95], rat B104 neuroblastoma [23] and differentiated PC12 cells [97]. In B104 neuroblastoma cells, the expression of LOX-1 was increment after treatment with oxidized LDL [23]. In endothelial cell, oxidized LDL increase the expression of LOX-1 and then lead to the increased intracellular ROS [98]. ROS activates the transcription factor such as nuclear factor KB that mediates gene expression for pro-inflammatory and adhesion molecules such as tumor necrosis factor responsible for apoptosis of vascular cells [98]. Oxidized LDL initiated the cleavage of procaspase-3 and also induced neurotoxicity by stimulating $Ca^{2+}$-dependent activation of mitogen-activated protein kinase (MAPK) signaling in primary striatal neuron [98]. LOX-1 is expressed in the cortex of rat brain and its expression has implication in neuronal apoptosis [27]. These evidences indicated that LOX-1 has implicated role in oxidative stress and neuronal cell apoptosis.

**3. Oxidized LDL and AD**

The antioxidant levels were reduced in the brain of AD patients suggesting the burden of oxidative stress [99]. In CSF of AD patients, the antibodies against oxidized LDL were significantly increased indicating the presence of oxidized LDL in AD [5]. LDL protein carbonyl content and protein oxidation marker were also found in AD plasma at higher levels than the control group [100].

There are reported that oxidation and nitration of protein to form carbonyl group and 3-nitrotyrosine is increased plasma from both hypercholesterolemia and dementia patients [101]. Moreover, oxidized LDL lipid intermediates such as isoprostanes and lipid hydroperoxides by free radical are more frequently in plasma

from hypercholesterolemia and AD subjects compared with control [102]. Peroxynitrite is a lipophilic and pass directly to plasma membrane via diffusion in the absence of transporter protein [103]. The rate of uptake of lipid hydroperoxide is 8-fold higher than parent lipid [104]. Thus, oxidized lipid can pass from periphery to CNS and induced neurotoxicity in neuron. LDL-L isolated from hypercholesterolemia patients with AD contain a higher level of lipid peroxidation [105]. In human microvascular endothelium cells (HMVECs), oxidized LDL increased the secretion of inflammation mediator such as TNF-α and IL-6 and decreased the membrane localization of the tight junction protein such as ZO-1 [105]. Thus, the oxidized LDL might be disrupted the microvascular endothelial cells and BBB dysfunction.

# CHAPTER III

# RESEARCH METHODOLOGY

This chapter consist of the methodology of this research including the materials, apparatus and experiments. The details of each part are given below.

**Chemicals and Reagents**

1. 0.25 % Trypsin/EDTA (REF No. 25200-072, Lot No. 1757812, Gibco, USA)

2. 1, 2-DI-(Dimethylamino) ethane (Cat No. 8920, Lot 17712503, Calbiochem)

3. 2', 7'-dichlorofluorescein diacetate (Cas No. 4091-99-0, Lot No.128K4145, Sigma-Aldrich, USA)

4. 3-(4, 5-dimethlthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (Code No. 0793 Lot No. 0880C145, Amresco Inc, USA)

5. β-Secretase Activity Assay kit (Fluorometric) (Cat No. ab65357, Lot No. GR213124-1, Abcam, UK)

6. Acetylcholine Iodide (Cat. No. A5751, Lot No. 094K1598, Sigma-Aldrich, USA)

7. Ammonium persulfate (Cat No. A3678, Lot No.104K0456, Sigma Aldrich, USA)

8. Absolute ethanol (AR grade RCI Labscan, Bangkok, Thailand)

9. Acrylamide/BisTM 37:5:1 (30:0:8), 40% (w/v) (Code 0254, Lot No. 2141C126, Amresco Inc, USA)

10. Amicron ® ultra centrifuge filter (REF UFC803008, Lot No. R4BA23528, Millipore, USA)

11. Goat polyclonal anti-AChE (Cat No. ab 31276, Abcam, UK)

12. Anti-human sAPPβ wild type (Cat No. 18957, Lot No. 1F-121, immunobiological laboratories, USA)

13. Anti-amyloid, β 1- 16, clone DE2 (Cat No. MAB5206, Lot No. NG1782829, Millipore, USA)

14. Anti-APP, N-terminus (Cat No. 07-667, Lot No. DAM1765523, Millipore, USA)

15. BCA protein assay Kit (Prod No. 23228, Lot No.QG218461, Thermo Scientific, USA)

16. Bromophenol blue (Cat No. 161-0404, Bio-Rad, USA)

17. Copper sulfate (Cat No. Lot No. Ajaxfinechem, Seven Hills, Australia)

18. CREB antibody (Cat No. 06-863, Lot No. 2144403, Millipore, USA)

19. Dimethylsulfoxide: DMSO (Cell culture grade, Cas No. 67-68-5, Lot No. 078K08921, Sigma,-Aldrich, USA)

20. Disodium hydrogen orthophosphate anhydrous (Lot No. 0912478, Ajaxfinechem, Australia)

21. Dulbecco's Modifide Eagle's Medium/Nutrient Mixture F-12 Ham without sodium bicarbonate (Cat. No. D8900 Lot No. 059K83061, Sigma-Aldrich, USA)

22. Dulbecco's Modifide Eagle's Medium/Nutrient Mixture F-12 Ham without phenol red (Cat No. D2906, Lot No. 021M8304, Sigma-Aldrich, USA)

23. Ethylenediamine tetraacetic acid disodium salt dehydrate (Cas No.6381-92-6 Lot No. SLBB8705V, Sigma-Aldrich, USA)

24. EnZChek® Caspase-3 Assay kit (Cat No. E13184 Lot No. 780223, Invitrogen, USA)

25. Fetal bovine serum (REF No. 10270-098, Lot No. 41A1513K, Gibco, USA)

26. Glycine (Cas No. 56406 Lot No. 1392C187, Amresco Inc, USA)

27. Hydrochloric acid (Bath No. 06060130, Lab-Scan, Thailand)

28. Methanol (AR grade RCI Labscan, Bangkok, Thailand)

29. Monoclonal Anti-β-actin (Cat No. A5441, Lot No. 121M4846, Sigma-Aldrich, USA)

30. β-mercaptoethanol (Cat No. 161-0710, Bio-Rad, USA)

31. Nuclear extraction kit (Cat No. 2900, Milipore, USA)

32. Penicillin and Streptomycin (REF No. 15140-122, Lot No. 15140-163, Gibco, USA)

33. Phospho-CREB antibody (Cat No. 05-807, Lot No. DAM1482729, USA)

34. Potassium chloride (Cas No. 7447407, J.T. Baker, Maxico)

35. Potassium dihydrogen orthophosphate (Cas No. 7778770, Fisher Scienticfic, UK)

36. Protease inhibitor cocktail (Cat No. P8340, Sigma-Aldrich, USA)

37. Peroxidase anti-rabbit IgG (Lot no. LV1636327, Milipore, USA)

38. Peroxidase anti-mouse IgG (Lot No. 2058947, Milipore, MA, USA)

39. Peroxidase anti-goat IgG (Cat No. ab 6741, Lot No. GR18798-17, Abcam, UK)

40. Specta/Por®dialysis membrane; MW: 6-8,000 (Reoder No. 132650, Lot No. 3243197, USA)

41. Secptra™ Multicolor Broad Range Protein Ladder (Cat No. 26634, Lot No. 00123891, Themo Scientific, USA)

42. Sodium bicarbonate (Cas No. 144-55-8, Lot No. S170701, Sigma-Aldrich Poland)

43. Sodium chloride (B/No. 1506196155, Ajax Finechem, Australia)

44. Sodium dodecyl sulfate (Lot No. 1205383, Ajax Finechem, Australia)

45. SuperSignal® west pico-chemiluminescence substrate (Prod No.34080, Lot NG176093, Thermo scientific, USA)

46. Thichloroacetic acid (Sigma, Missouri, USA)

47. Thiobarbituric acid (Sigma, Missouri, USA)

48. Tris (hydroxymethy) amino-methane, ultra pure grade (Cas No. 77-86-1, Research organics, USA)

49. Trypan blue (Cell culture grade, Cas No. 72-57-1, Lot No. 123K53301, Sigma-Aldrich, USA)

48. Tween - 20 (Cas No. 9005-64-5, Lot No. Bio Basic Inc, Canada)

**Cell line and Plasma**

1. Human SH-SY5Y neuroblastoma cell lines (CRL-2266™, ATCC, USA)

2. Healthy human plasma (Blood Blank, Naresuan University Hospital, Phitsanulok, Thailand)

**Instruments**

1. Centrifuge Bottles Polycarbonate (Reoder No. 355603, Lot No. P 10603, Beckman Instrument, Inc., USA)
2. Beckman Coulter Optima L-80 XP Ultracentrifuge (Beckman Instrument, Inc., USA)
3. Type 90 ti rotor (Beckman Instrument, Inc.,USA)
4. PH meter (S20K, Mettler-Teledo GmbH, Schwerzenbach, Switzerland)
5. Autoclave (HA-300P, Hirayama Manufacturing Corporation, Saitama, Japan)
6. $CO_2$ Incubator (Forma series II, Thermo Fisher Scientific Inc., USA)
7. Laminar flow hood (Heal force®, HF safe 1200/c+, Shanghai, China)
8. Microplate Spectrophotometor (Multimode detector DTX 880, Beckman Instrument, Inc., USA)
9. Semi-Dry blotting (Bio-Rad, USA)
10. Vertical gel electrophoresis (Model Mini-PROTEIN Tetra Cell, Bio-Rad laboratory, Philadephia, USA)
11. ChemiDocTM XRS+ with image laboratories software (Bio-Rad, Philadephia, USA)

**Methodology**

**1. Preparations of LDL and oxidized LDL**

**1.1 Isolation of human LDL**

Human LDL was isolated from plasma obtained from Blood Bank, Naresuan University Hospital by density gradient ultracentrifugation. As shown Figure 6, plasma was adjusted to a density of 1.20 g/ml with solid potassium bromide (KBr). This high density plasma (2.8 ml, d =1.20 g/ml) was loaded to the bottom of ultracentrifuge tube (10.4 ml tube) and then overlaid with NaCl salt density 1.006 g/ml (6.6 ml) containing 0.05 % EDTA. These samples were centrifuged at 65,000 rpm, 15°C for 3 h. The yellow-orange LDL fraction appearing in the middle of the tube (1.019-1.063 g/ml) was collected. LDL was dialyzed for 24 h at 4 °C with 500 volumes of 1XPBS containing 10 μM EDTA to prevent LDL oxidation. Buffer was changed three times during dialysis. All preparations of LDL were filtered through a

0.22-μM filter and stored at 4 °C under $N_2$ gas to prevent oxidation of LDL and keep for up to 1 week. Protein concentration in the LDL preparation was determined by BCA assay kit.

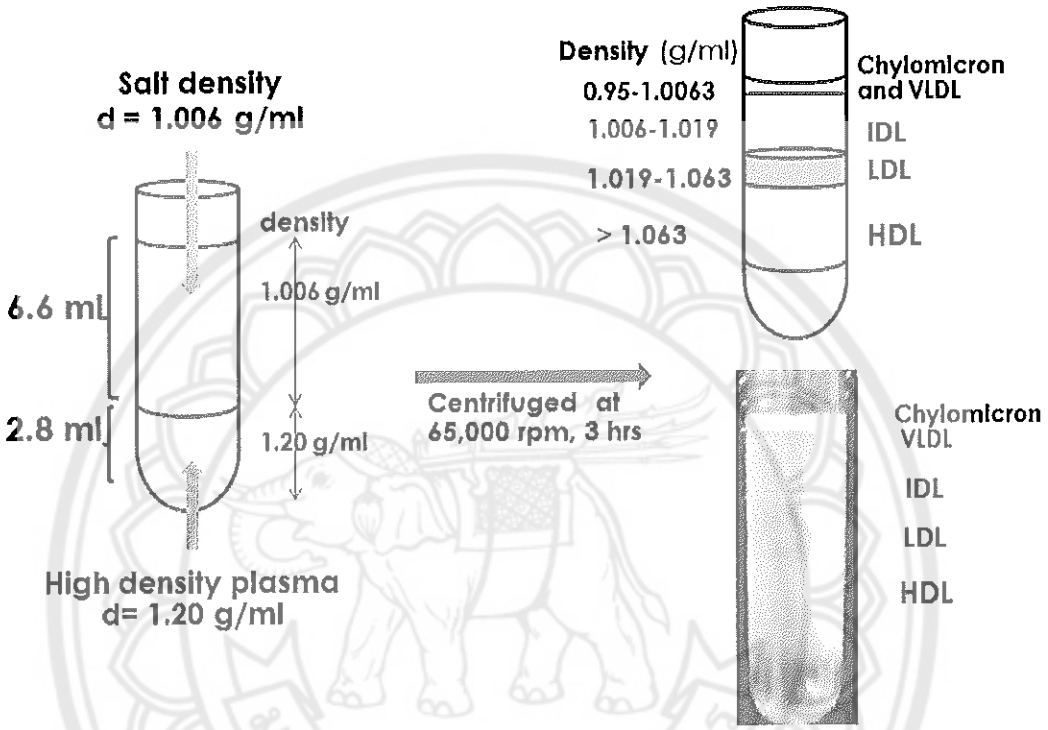



**Figure 6 Schematic of LDL isolation by density gradient ultracentrifugation**

### 1.2 Preparation of oxidized LDL

LDL concentration was adjusted to 1 mg/ml with 1xPBS and oxidized by 10 μM $CuSO_4$ at 37 ° for 1 h to prepare mildly oxidized-LDL (mox-LDL) and for 24 h to prepare fully oxidized LDL (fox-LDL). The oxidation reaction was terminated by the addition of 0.3 mM EDTA. Degree of LDL oxidation was followed by monitoring the increased formation of conjugated dienes by measuring the absorbance at 234 nm. The final level of oxidation was assessed by measuring the level of thiobarbituric acid-reactive substances (TBARS) using malondialdehyde (MDA) generated from 1, 1, 3, 3, -tetramethoxypropane as a standard. Before using, EDTA and $CuSO_4$ were removed by filtering the LDL sample through a 30 kDa cut-off amicron tube. The protein concentration of oxidized LDL was assessed again by BCA assay kit.

**1.3 Thiobarbituric reactive substance (TBARS) assay**

Oxidized LDL (mox- and fox-LDL at 1 mg/ml was mixed with TBARS reagent containing 1.4 % trichloroacetic acid (TCA), 0.4 % thiobarbituric acid (TBA) and 8% HCl (1:2:1). The mixture was incubated at 90 °C for 1 h, then cooled on ice for 10 min and centrifuged at 5,000 rpm for 5 min at 4 °C. The supernatant was collected and fluorescence intensity was determined at 535 nm exitation and 595 nm emission by microplate reader. The MDA standard curve was prepared using 1, 1, 3, 3-tetraethoxypropane.

**1.4 Measurement of conjugated dienes**

The oxidation of unsaturated fatty acid side-chains was accompanied by the formation of conjugated diene structures that absorbed ultraviolet light at 234 nm wavelength. The LDL, mox & fox LDL (1 mg/ml) was measured to determine the formation of dienes with UV-absorption at 234 nm by using UV plate and the absorbance was determined by microplate reader. The formation of conjugated dienes in oxidation of LDL was form to double bond which indicated that one molecule of lipid peroxidation in LDL particle. The level of conjugated dienes in oxidized LDL was calculated as a ratio of native LDL.

**2. Study the effects of LDL and oxidized LDL on differentiated SH-SY5Ycells**

**2.1 Cell culture and preparation**

SH-SY5Y Human neuroblastoma cell line was routinely cultured in DMEM/F-12 supplement with 10% FBS and 1% penicillin-streptomycin. Cell cultures was plated at a density of $1\times10^6$ cells/$cm^2$ in 75 $cm^3$ flasks and then incubated at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$. The culture medium was changed every 3-4 days. All experiments were carried out by using cells between 10 to 30 passages to ensure cell uniformity and reproducibility. The cells were seeded into 96 well plates at a density of 20,000 cells/well for cytotoxicity, intracellular ROS and AChE activity assay. They were seeded in the culture medium and allowed to attach for 24 h and then differentiated to neuron like cells by replacement the medium with low serum culture medium (2 % FBS) containing retinoic acid (10 μM) for 6 days. After that, cells were replaced with low serum culture medium without retinoic acid for 24 h.

**2.2 Determination of cell viability by MTT assay**

Cell viability assay is based on the alteration of MTT to the purple formazan crystal by dehydrogenase activity in viable cell. SH-SY5Y cells were incubated with LDL or oxidized LDL in DMEM/F-12 without FBS and retinoic acid at various concentrations (10-200 μg/ml) for 1, 2, 4 and 24h. At the end of treatment, 20 μl of MTT (5 mg/ml in PBS) was added to each well and incubated at 37 °C for 2 h. The medium was discarded and 200 μl of DMSO: ETOH (1:1) was added to dissolve the purple formazan crystals. Light absorption was measured at 595 nm using a microplate reader. Cell viability was expressed as a percentage of the control (untreated) cells.

**2.3 Determination of lactase dehydrogenase (LDH) activity**

Lactase dehydrogenase (LDH) is a soluble enzyme located in the cytosol. Its release into the surrounding culture medium upon cell damage can indicate cell necrosis. In this study, LDH released from differentiated SH-SY5Y cells was measured after LDL and oxidized LDL treatment for 24h. The principle of LDH activity assay is based on the ability of LDH to catalyze NADH to $NAD^+$ and $H^+$ by oxidation of pyruvate to lactate. The disappearance of NADH in the presence of sodium pyruvate was monitored with a microplate reader. Decrease in the absorbance at 340 nm is linked directly to the increasing quantity of LDH released from cells to the culture media. The results were normalized by total number of viable cells and presented as percentage of the control (untreated) cells.

**2.4 Measurement of intracellular reactive oxygen species (ROS)**

Reactive oxygen species (ROS) production in the living cell was detected by dichloro-dihydro-fluorescein diacetate (DCFH-DA). DCFH-DA can pass through the cell membrane and is hydrolyzed by intracellular esterase to DCFH which is rapidly oxidized to highly fluorescent diclorofluorescein in the presence of ROS. Differentiated SH-SY5Y cells were pre-incubated with FBS-free DMEM/F-12 (without phenol red) containing 10 μM DCFH-DA for 30 min and washed with 1xPBS after removing the medium. Then, the cells were treated with various concentration of LDL or oxidized LDL for 1, 2, 4 and 24 h. The fluorescence intensity was determined at 485 nm exitation and 530 nm emission using a fluorescence microplate reader. The

fluorescent signal was normalized by total number of viable cells and presented as percentage of the control (untreated) cells.

**2.5 Measurement of cellular acetylcholinesterase (AChE) activity**

Differentiated SH-SY5Y cells were treated with LDL and oxidized LDL at various concentrations (10-200 μg/ml) for 1, 2, 4 and 24 h. At the end of the treatment, the medium was removed and the cells was lysed with 50 μl of lysis buffer containing 15 mM Tris (pH 7.4), 150 mM NaCl and 1% triton X-100. AChE activity in cell lysate was determined by an enzymatic assay using acetylthiocholine iodide (ATChI) at 0.5 mM as a substrate. The formation of yellow compound from the reaction of 5, 5'-dithio-bis (2-nitrobezonic acid) (DTNB, 1.5 mM) with thiocholine released by the enzymatic hydrolysis of ATChI was determined at 405 nm. Neostigmine, a known AChE inhibitor was used to verify the assay. The AChE activity was normalized to the total amount of viable cells and presented as a percentage of the control (untreated) cells.

**2.6 Measurement of caspase-3 activity**

The cells were plated in a 6-well plate at $1\times10^6$ cells per well in DMEM/F-12 containing 10% FBS. After SH-SY5Y cell differentiation, the culture medium was replaced with free serum medium and the cells were treated with oxidized LDL or LDL at 50 and 100 μg/ml for 24 h. At the end of treatment, the differentiated cells were harvested by lysis buffer following the instruction. The amount of protein was determined by BCA assay kit. Caspase-3 activity of cell lysate was determined using Caspase-3 assay fluorimetric kit. According assay to the instruction, caspase-3 substrate (Z-DEVD-AMC) was mixed in 2x reaction buffer to 300 μM. Cell lysate (50 μl) and substrate (50 μl) was mixed in a 96-black well plate at 37 °C for 1 h. The fluorescence of the cleavage product was measured in a microplate spectrofluorometor at excitation 360 nm and emission 535 nm. The AC-DEVD-CHO inhibitor was used to verify the assay. Caspase-3 activity was calculated using 7-amino-4-methylcoumarin (AMC) as standard curve.

**2.7 Measurement of β-secreatase activity**

The cells were plated in a 6-well plate at $2\times10^6$ cells per well with DMEM/F-12 containing 10% FBS. After SH-SY5Y cell differentiation, the culture mediums were replaced with free serum medium. Differentiated cells were treated

with each type of oxidized LDL or LDL at 50 μg/ml for 24 h. At the end of treatment, the differentiated cells were harvested by lysis buffer. The amount of protein was determined by BCA assay kit. Beta secretase in cell lysate was determined by β-secretase assay kit. According to the instruction, cell lysate (50 μl) was mixed with 50 μL 2X reaction buffer in a 96-black well plate and pre-incubated 5 min before adding 2μl substrate then gently mixed and incubated in the dark at 37°C for 1 h. The fluorescence of the cleavage product was recorded at excitation 360 nm and emission 535 nm. β -Secretase inhibitor and reconstructed active β-secretase were used to confirm the assay. The β-secretase activity was normalized by protein concentration and expressed activity as fluorescence intensity/μg protein.

**2.8 Western blotting**

**2.8.1 Expression of AChE**

Expression of AChE was determined by western blotting. SH-SY5Y cells was plated in 60-mm petri dish at $1x10^6$ cells per dish with DMEM/F-12 supplemented with 10% FBS. After cell differentiation, the culture medium was replaced with FBS-free DMEM/F-12 and then treated with LDL or oxidized LDL at various 50 and 100 μg/ml for 24 h. The cells were lysed by lysis buffer, & protein concentrations were determined by BCA assay kit. The cell lysate (40 μg) was loaded and separated on 12 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and finally transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with 10% skim milk in PBS at room temperature for 1 h to block non-specific binding protein. After being washed with PBS containing 0.05 % Tween-20, the membrane was incubated with goat polyclonal anti-AChE (ab 31276; 1:1,000) overnight at 4°C and then incubated with secondary antibody (1:5,000) conjugated horseradish peroxidase for 1h. The protein of interest was detected by SuperSignal® west pico-chemiluminescence substrate. The chemiluminescence signal was visualized with ChemidocTMXRS and analyzed with Image LabTM software. The intensity of β-actin was used as a loading control.

**2.8.2 Releasing of sAPPβ, sAPPα and sAPP**

SH-SY5Y cells was seeded into a 60-mm petri dish at $1x10^6$ cells per dish with DMEM/F-12 supplemented with 10% FBS. Differentiated SH-SY5Y cells were treated with LDL or oxidized LDL at 50 μg/ml. After 24 h of

treatment, the 3 ml of culture medium was collected and centrifuged to remove cell debris at 3600 rpm for 10 min. The 2.5 ml culture medium was then collected and concentrated by centrifugation using 30 kDa cut off amicron tube until the volume reached 100 µl. Equal volume of the concentrated medium was loaded and separated on 8 % SDS-PAGE and transferred onto PVDF membrane. The protein of interest was determined by immunoblotting using the primary antibodies specificity on each type of sAPP including antibodies used to detect sAPPβ and sAPPα were rabbit polyclonal antibody anti-sAPPβ wild type (against residues ISEVKM) (1:500) and mouse monoclonal anti-amyloid (beta 1-16, clone DE2) (1:200), respectively. It should be noted that all antibodies used in this study were tested and showed no interaction with proteins in LDL and oxidized-LDL particles.

For measuring the level of full length APP in cell lysate, cells were lysed by lysis buffer (Tris-HCl, pH 7.4, contain 0.5 % SDS and 1% of protease inhibitor cocktail). The full length APP in cell lysate was also measured by western blotting using anti-APP (N-terminus) (1:1000) and equal sample loading was verified using β-actin.

**2.8.3 Level of phosphorylated CREB and total CREB**

Oxidative stress found decreased pCREB within 1 h treatment, whereas the loss of total CREB protein was observed 2 h after $H_2O_2$ treatment. SH-SY5Y neuroblastoma cells ($2\times10^6$ cells) were seeded on a 60-mm culture dish. After LDL and oxidized LDL at 15 and 60 min treatment, cells were lysed using nuclear extraction kit. From the instruction, cells were disrupted by incubating the cells pellet in cytoplasmic lysis buffer containing 0.5mM DTT, 1% phosphatase inhibitor and 1% protease inhibitor for 15 min on ice and then centrifuged at 250 g for 5min. The extraction assay are firstly cell lysis step, the pellet was collected and added the cytoplasmic lysis buffer containing 1% phosphatase inhibitor and 1% protease inhibitor. The pellet was draw and suspended by gauge needle (27 gauge) approximately 5 times and then centrifuge at 8000 x g for 20 minute at 4 °C. The supernatant in this step contains the cytosolic portion of cell lysate was transferred to a fresh tube. Finally, the remaining pellet that contained the nuclear portion of the cell lysate was added to the nuclear extraction buffer containing 1% phosphatase inhibitor and 1% protease inhibitor and then draw by gauge needle (27 gauge) approximately 5

times. The nuclear suspension was gently agitate using orbital shaker for 30 min at 4 °C and then the nuclear suspension was centrifuged at 16,000 x g for 5 min. The supernatant in this step is nuclear extract. The protein concentrations was determined by BCA assay. Nuclear extract (20 µg) was loaded and separated in 12 % SDS-polyacrylamide gels and transferred to PVDF membrane. The protein of interest was determine by immunoblotting using anti-CREB (1:1000) and phospho-CREB (1:1000) following the same step in 2.8.1

**3. Statistical analysis**

Data from all assays were expressed as mean±standard errors of mean (SEM) from at least three different cell passages. The data were analyzed by analysis of variance (ANOVA) and followed by with a LSD test. Differences were considered to be significant when $p \leq 0.05$. Correlation between variables were assessed by Pearson's correlation, $p \leq 0.05$ was considered significant.

# CHAPTER IV

# RESULTS AND DISCUSSION

The present study aimed to investigate the effect of two oxidized LDL preparations; mildly oxidized (mox-LDL) and fully oxidized-LDL (fox-LDL) on neurotoxicity and cellular oxidative stress. The mechanisms of cell toxicity, necrotic and apoptotic death were also determined by LDH release and caspase-3 activity, respectively. Cellular activity and expression of AChE as consequences of those LDL treatments were also determined. The effects of subtoxic concentration of oxidized LDL were tested on APP processing by monitoring sAPPβ and sAPPα and BACE-1 activity. Finally, the phosphorylation of CREB signaling was explored. The detail of results and discussion are given below.

**Characteristics of LDL and Oxidized LDL**

The composition of oxidized LDL is dependent on both nature and concentrations of the oxidative and agent condition of the exposure. Increased oxidation of LDL level was positively correlated with the severity of acute coronary [106]. It was reported that oxidized LDL was induced by copper ions characterized and similar to levels of oxidation in vascular cell [106]. Copper also generated a spectrum degrees of LDL oxidation depending on time of exposure [107]. In macrophage, oxidized LDL induced cellular response dependent on the degree of LDL oxidation [108]. Because degree of LDL oxidation seems important for cellular response, two oxidized LDL preparations were generated.

In this study, native LDL was oxidized by 10 μM $CuSO_4$ at 37 ° C for 1 h and 24 h to prepare for mildly oxidized LDL (mox-LDL) and fully oxidized LDL (fox-LDL), respectively. The degree of LDL oxidation was assessed by measuring the lipid peroxidation and conjugated diene formation. The oxidation of unsaturated fatty acid side-chain was accompanied by the formation of conjugated diene formation structures that absorbed ultraviolet light at 234 nm wavelength. Conjugated diene levels in mox- and fox-LDL were approximately 2 times and 10 times higher than native LDL,

respectively (Table 4). Lipid peroxidation product in oxidized LDL particle was also presented as malondialdehyde (MDA) using thiobarbituric reactive substance (TBAR) assay. The MDA levels in fox-, mox-, & native LDL were 10.3, 3.8 and 0.03 μM (Table 4).

Oxidized LDL contained lipid peroxidation products such as 4-hydroxynonenal (4-HNE), lipid hydroperoxides (LOOH), & oxysterol [26, 93]. These products are cytotoxicity in various cell types [24, 26, 108, 109]. The relative toxicities of lipid peroxidation formed on oxidized LDL were 7β-hydroperoxycholesterol > 7β-hydroxycholesterol = 4-hydroxynonenal > 7-ketocholesterol > 5α, 6α-epoxycholesterol in human fibroblast [109]. The toxicity of oxidized LDL is also strongly like between the LOOH concentration within the oxidized LDL particles [110]. So, it is possible that 7β-hydroperoxycholesterol is predominantly responsible compound in oxidized LDL. Moreover, protein oxidation have been appeared on oxidized LDL [110]. The alteration of the surface charge on the apolipoprotein B100 was occurred after oxidation of LDL [110]. The electrophoretic mobility of ApoB100 were 0.46 and 1.46 after oxidation for 45 min and 24 h respectively, while native LDL was 0.30, relative to albumin [110]. The higher concentrations of lipid peroxidation product on oxidized LDL might be different effect on cellular response in neuron cell. Thus, further study aimed to investigate the effect and mechanism of neurotoxicity of oxidized LDL on human SH-SY5Y neuroblastoma cell line.

**Table 4 The characteristic of LDL and oxidized LDL preparations**

| LDL sample (1 mg/ml) | MDA (μM) | Conjugated diene (fold of native LDL) |
|---|---|---|
| **native LDL** | 0.03 ± 0.01 | 1.0 |
| **mox-LDL** | 3.76 ± 0.34 | 1.50 ± 0.10 |
| **fox-LDL** | 10.26 ± 0.92 | 13.90 ± 0.20 |

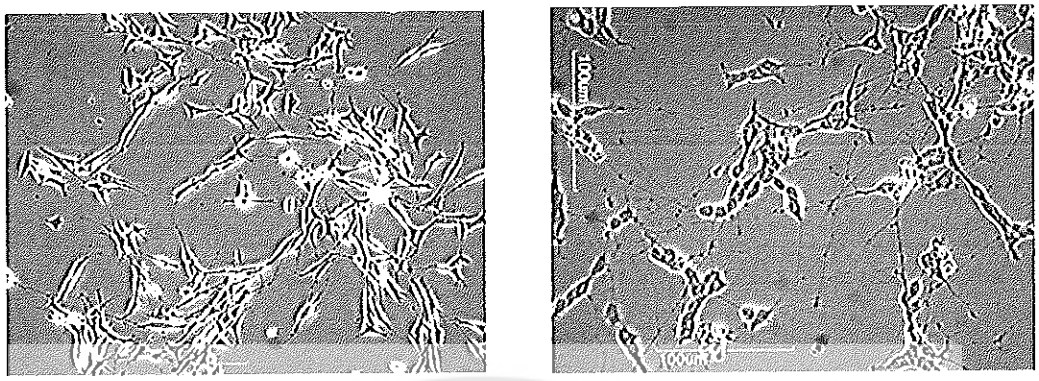
**Note:** Data present mean ± SEM of eleven LDL preparations

**Effect of LDL and oxidized LDL on differentiated SH-SY5Y cells**

**1. Effect of LDL and oxidized LDL on the viability of differentiated SH-SY5Y cells**

Antibodies against oxidized LDL and cholesterol metabolite were increased in the CSF of AD patients [5, 111]. Thus, oxidized LDL might be associated with the development of AD pathogenesis. Although, there are certain number of studies demonstrating the link between LDL or oxidized LDL and AD, there is limited information of which AD pathological processes were affected by these lipoproteins. Thus, this study hypothesized that oxidized LDL has neurotoxicity and we also investigated the mechanism of neurotoxicity of oxidized LDL on human SH-SY5Y neuroblastoma cell line.

The SH-SY5Y cells are widely used as an *in vitro* model to study the neuronal function related to neurodegeneration, & neuroadative processes, neurotoxicity, & neuroprotection. SH-SY5Y cell is a subline of SK-N-SH cells that were originally derived from bone marrow biopsy of neuroblastoma patients [112]. They exhibit neuronal marker enzymes (tyrosine and dopamine-β-hydroxylase) and also express opioid receptor, muscarinic receptor and nerve growth factor. Differentiation of SH-SY5Y resulted in the biochemical, ultrastructural, morphological and electrophysiological properties similar to mature neuron (Figure 7). The RA is mostly used to differentiate the SH-SY5Y cells. RA is an active metabolite of vitamin A. It plays a role to regulate the proliferation of many cells such as embryonic carcinoma cells, hematopoietic and neuronal precursors [113]. RA can bind to nuclear receptor family such as RA receptor and the retinoid X receptor and then bind to RA response element resulting in transcriptional activation [113]. Differentiated SH-SY5Y cells induced by RA express the APP protein family [114]. In addition, this cell expresses choline acetyltransferase, acetylcholine esterase and cholinergic receptor [115, 116, 117].



Non-differentiated SH-SY5Y cells Differentiated-SH-SY5Y cells

**Figure 7 The morphology of human SH-SY5Y neuroblastoma cells**

In this study, differentiated SH-SY5Y cells were treated with mox-, fox-LDL and native LDL at 10-200 μg/ml for 1, 2, 4 and 24 h. The cytotoxicity was measured by MTT assay. At 1 and 2 h incubation, high concentration (100-200 μg/ml) of all LDL samples slightly decreased the viability of SH-SY5Y cells but these changes did not reach the significant level (Figure 8, 9). After 4 h treatment, only fox-LDL at 200 μg/ml significantly reduced viability of cells compared to control and native LDL (Figure 10). At 24 h treatment, both mox- and fox-LDL at 100 and 200 μg/ml clearly decreased cell viability (Figure 11). LDL at 200 μg/ml also significantly reduced cell viability (Figure 11). These data suggest that oxidized LDL exhibited neurotoxicity and this toxic effect depended on degree of oxidation. It should be noted that $Cu^{2+}$ ions and EDTA were removed from the LDL samples before treating with cells, & expected concentration of $Cu^{2+}$ (1 μM) remaining in sample was tested and showed no effect on cell viability. These results therefore indicate a neurotoxic effect of oxidized LDL.

Previous study determined the formation of lipid hydroperoxides (LOOH) within oxidized LDL particle and found that LOOH concentration was strongly linked to neuronal death [110]. Our study support the finding that cytotoxic effect of oxidized LDL was depending on degree of LDL oxidation. The lipid peroxidation products could be involved in neuronal cell death. Lipid peroxidation products such as 4-HNE and oxysterols mediated the oxidative stress by causing impairment of mitochondria function [118], induced apoptotic process, & increased intracellular $Ca^{2+}$ [119]. In

addition, hydroperoxides and aldehydes can react and disrupt cellular components such as protein and DNA [110]. Thus, lipid peroxidation products could mediate various cells signaling to induce neuronal death.

Oxidized LDL has been reported to induce neuronal cell death by increasing intracellular ROS and $Ca^{2+}$, depleting cellular glutathione, & elevating caspase activation [16, 23, 25, 120]. In addition, 27- OHC, a major oxysterols in periphery, which is crosses the BBB when the BBB dysfunction. These oxysterol mediated neuron death by depleting intracellular glutathione [16]. The uptake of oxidized LDL particle into neurons was also thought to be involved with oxidized LDL receptors. Binding to LOX-1, oxidized LDL receptor, expressing in neuroblastoma cells [23] and toll like receptor-4 (TLR-4) expressing in dorsal root ganglionic cells [24] could be the possible pathways of oxidized LDL-induced neuronal death. Next experiment aimed to evaluate the effect of oxidized LDL on cellular oxidative stress by monitoring the level of intracellular ROS.

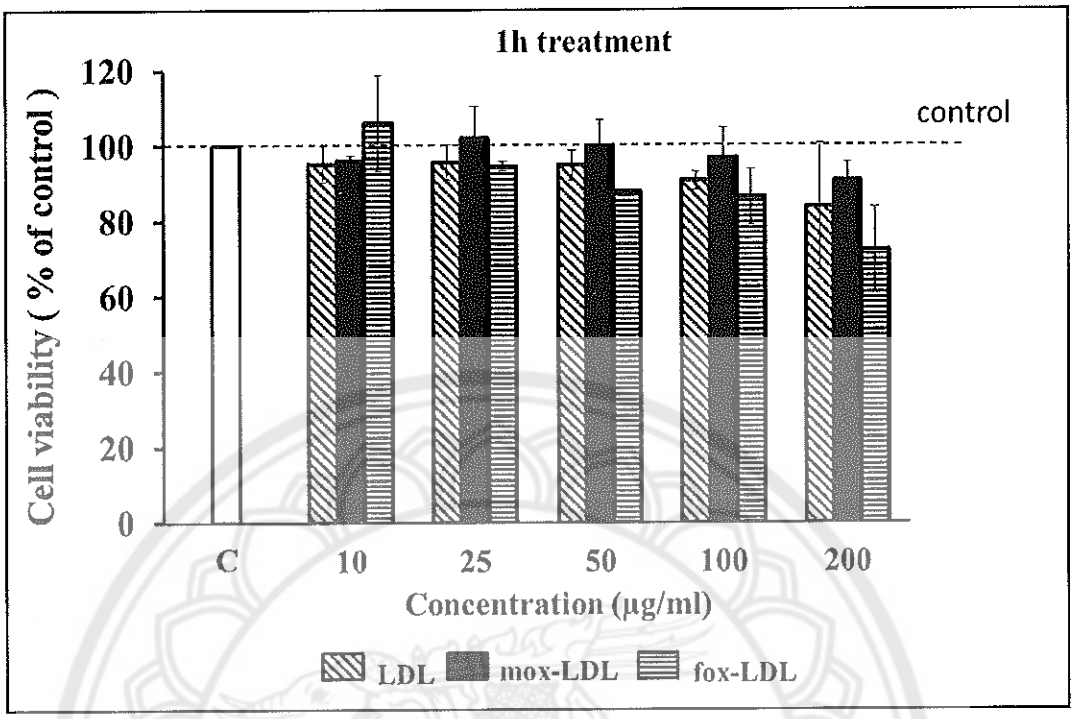



**Figure 8 Effect of LDL and oxidized LDL on cell viability after 1 h treatment**

**Note:** SH-SY5Y neuroblastoma cells were treated with 10, 25, 50, 100 and 200 µg/ml of LDL preparations for 1 h. Cell viability was assessed by MTT assay and calculated as percent of control or untreated cells. Data present as mean±SEM from at 5-7 independent experiments

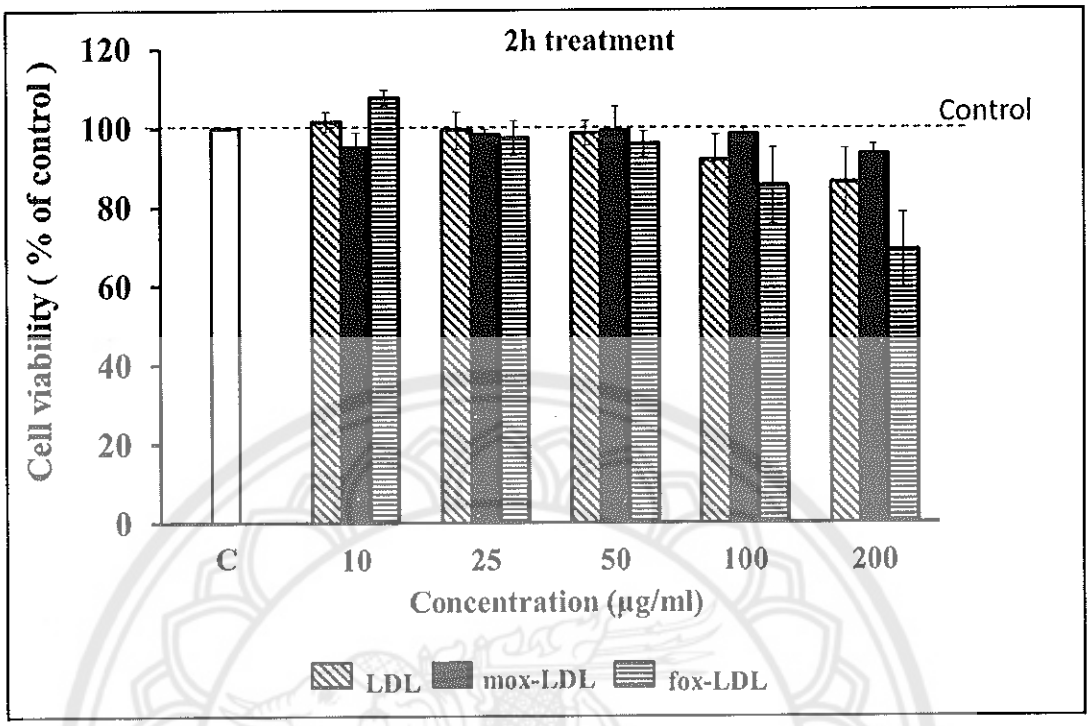



**Figure 9 Effect of LDL and oxidized LDL on cell viability after 2 h treatment**

**Note:** SH-SY5Y neuroblastoma cells were treated with 10, 25, 50, 100 and 200 µg/mL of LDL preparations for 2 h. Cell viability was assessed by MTT assay and calculated as percent of control or untreated cells. Data present as mean ± SEM from at 5-7 independent experiments

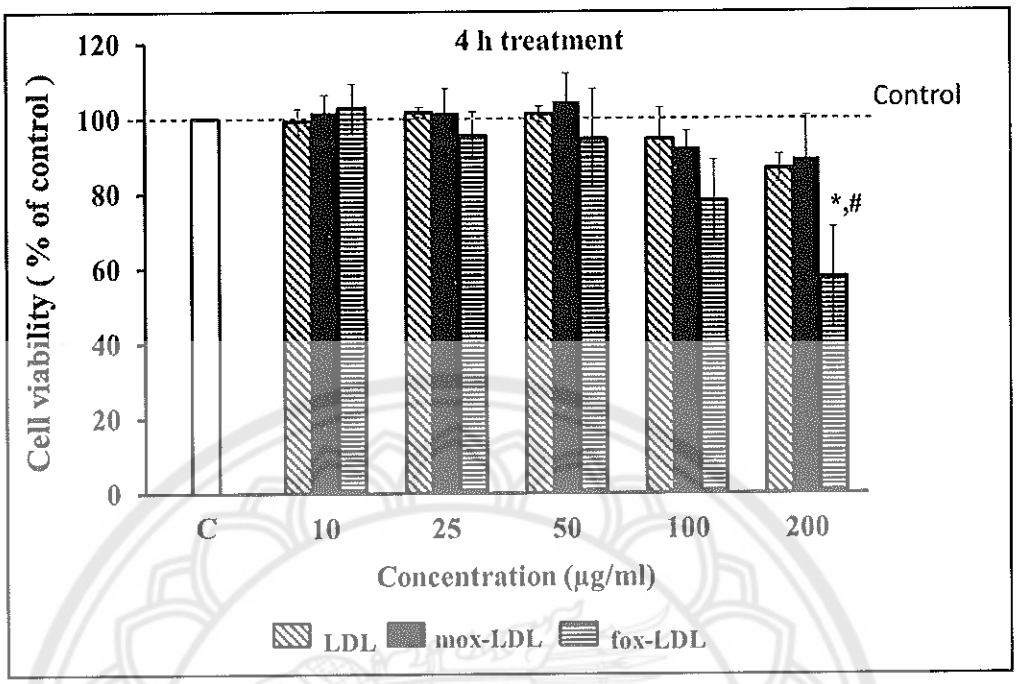



**Figure 10 Effect of LDL and oxidized LDL on cell viability after 4 h treatment**

**Note:** SH-SY5Y neuroblastoma cells were treated with 10, 25, 50, 100 and 200 µg/ml of LDL preparations for 4 h. Cell viability was assessed by MTT assay and calculated as percent of control or untreated cells. Data present as mean ± SEM from at 5-7 independent experiments. (* $p \leq 0.05$ versus control, # $p \leq 0.05$ versus LDL)

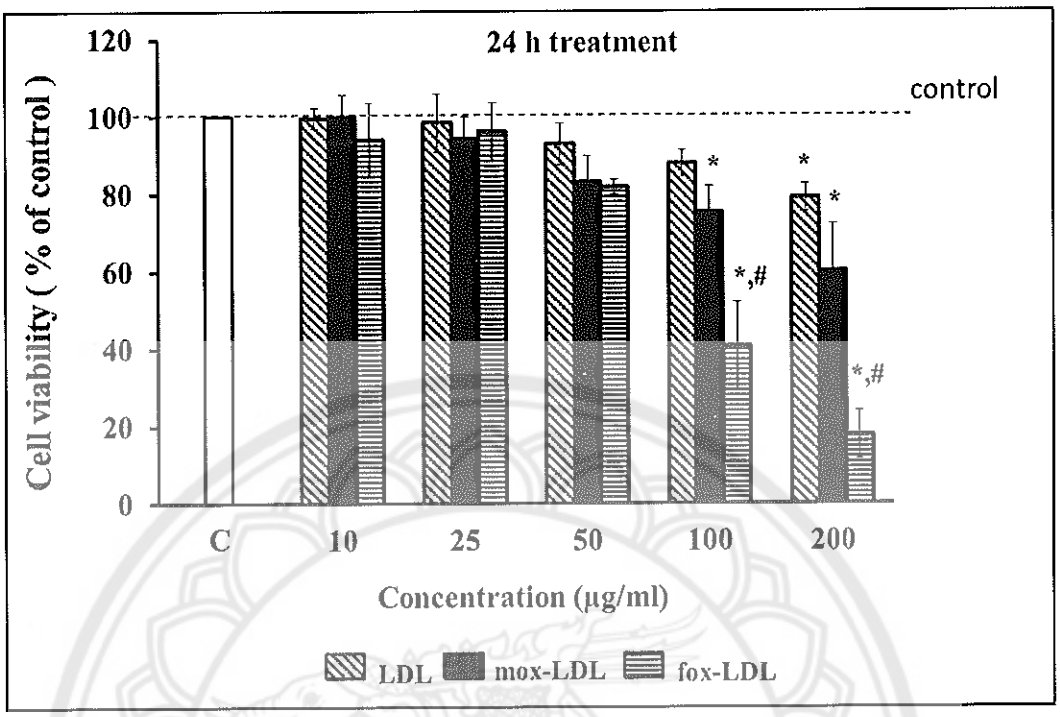



**Figure 11 Effect of LDL and oxidized LDL on cell viability after 24 h treatment**

**Note:** SH-SY5Y neuroblastoma cells were treated with 10, 25, 50, 100 and 200 µg/mL of LDL preparations for 24 h. Cell viability was assessed by MTT assay and calculated as percent of control or untreated cells. Data present as mean±SEM from at 5-7 independent experiments. (* $p \leq 0.05$ versus control, # $p \leq 0.05$ versus LDL)

**2. Effect of LDL and oxidized LDL on intracellular ROS**

Oxidative stress can damage the macromolecules including protein, lipid, & DNA [70]. Lipid peroxidation products induced by oxidative stress inside the neurons can cause cell death. The effect of oxidized lipoprotein oxidation on neuronal cell are less investigated. This study aimed to evaluate the effect of oxidized LDL on neuronal oxidative stress by measuring the intracellular ROS.

SH-SY5Y cells were treated with various concentrations of oxidized LDL (10-200 μg/ml). The result showed that intracellular ROS was increased at 1, 2, 4 and 24 h incubation (Figure 12-15) after treating cells with mox- and fox-LDL. ROS levels were much higher at 24 h treatment with fox-LDL comparing to mox-LDL (Figure 15). Native LDL at all treatment conditions did not change the levels of intracellular ROS. It should be noted that $Cu^{2+}$ ions were removed from the LDL samples before treating with cells, & expected concentration of $Cu^{2+}$ (1 μM) remaining in sample was tested and showed no effect on intracellular ROS. According to these data, the relationships between intracellular ROS levels and cell viability was examined and the result showed inverse correlation between these two variables (Figure 16). These results indicate the ability of oxidized LDL to induce cellular oxidative stress and consequently cause neurotoxicity.

In previous study, oxidized LDL has showed to increase the intracellular ROS in primary rat striatal cultured neuron and motor neuron [26, 121]. From our study, the intracellular ROS level were more increased by fox-LDL than mox-LDL. (Figure 12-15). Thus, the mechanism of oxidized LDL to induce intracellular ROS production might be possibly involved with lipid peroxidation products containing in lipoprotein particles. 4-Hydroxynanonal (HNE), lysophospholipids and oxysterol were found in oxidized LDL complex at relatively high concentration and possibly induced oxidative stress and mediated neuronal cell death [25, 110, 122]. HNE is mostly extensive studied and is a highly electrophilic molecule [123]. It easily reacts with low molecular weight compound such as glutathione, protein or DNA [123]. It composed of three major chemical reactivity group including the aldehyde group, the C=C double bond and hydroxyl group [123]. These functional groups are found participating in chemical reaction with other molecule [123]. It is possibly that lipid

peroxidation product is a reactive compound and generate directly or exert the cellular response in consequence the increase intracellular ROS.

Moreover, the receptor mediated endocytosis of oxidized LDL into cells might be one of the pathway possible to increase ROS. Oxidized LDL could bind to the LOX-1 receptor in neuroblastoma cells and TLR-4 receptor in dorsal root ganglionic cells [23, 24]. In striatal neuron, oxidized LDL can be internalized into neuronal cells [110] and disturbance the mitochondria [118]. It is possibly when oxidized LDL was internalized into cell and the lipid peroxide products contain in oxidized LDL complex might be responsible as reactive compounds in cellular response by depleted the glutathione, caused disruption the mitochondria and induced intracellular ROS inside the cell, & finally led to cell death. Thus, lipid peroxidation product of oxidized LDL or oxidized LDL particle might be as reactive oxidant and mediate the increase intracellular ROS.

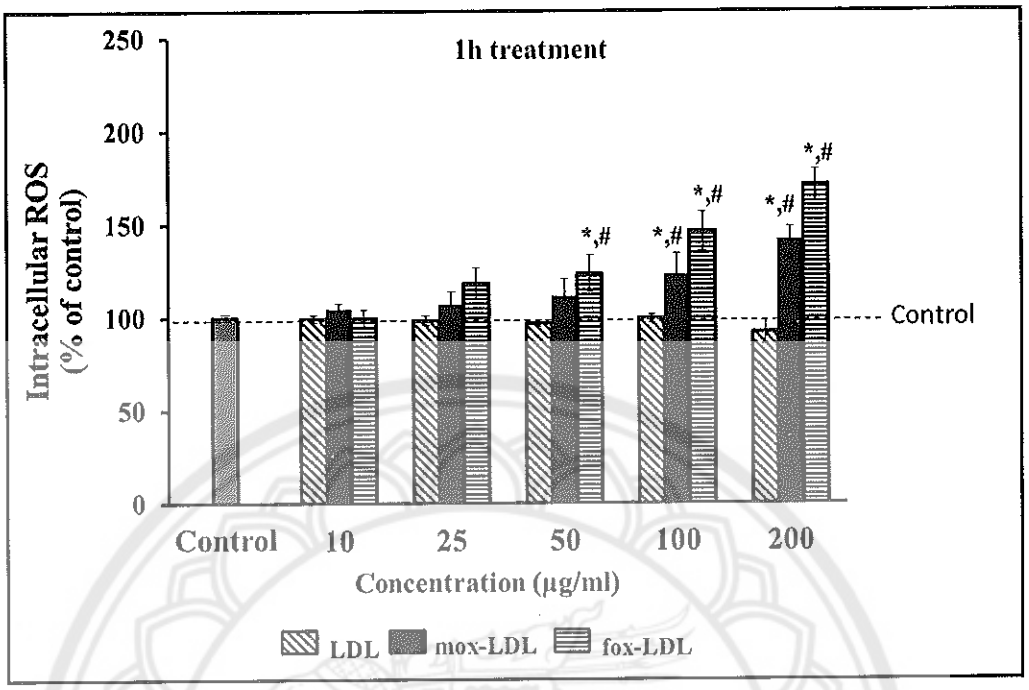



**Figure 12 Effect of LDL and oxidized LDL on intracellular ROS at 1 h treatment**

**Note:** SH-SY5Y neuroblastoma cells were treated with 10, 25, 50, 100 and 200 µg/ml of LDL preparations for 1 h. Fluorescence of DCF measured at 480 nm excitation and 530 nm emission was refered to intracellular ROS levels. Data present as mean ± SEM from at least five independent experimen ts. (* $p \leq$ 0.05 versus control or untreated cells, # $p \leq 0.05$ versus LDL)

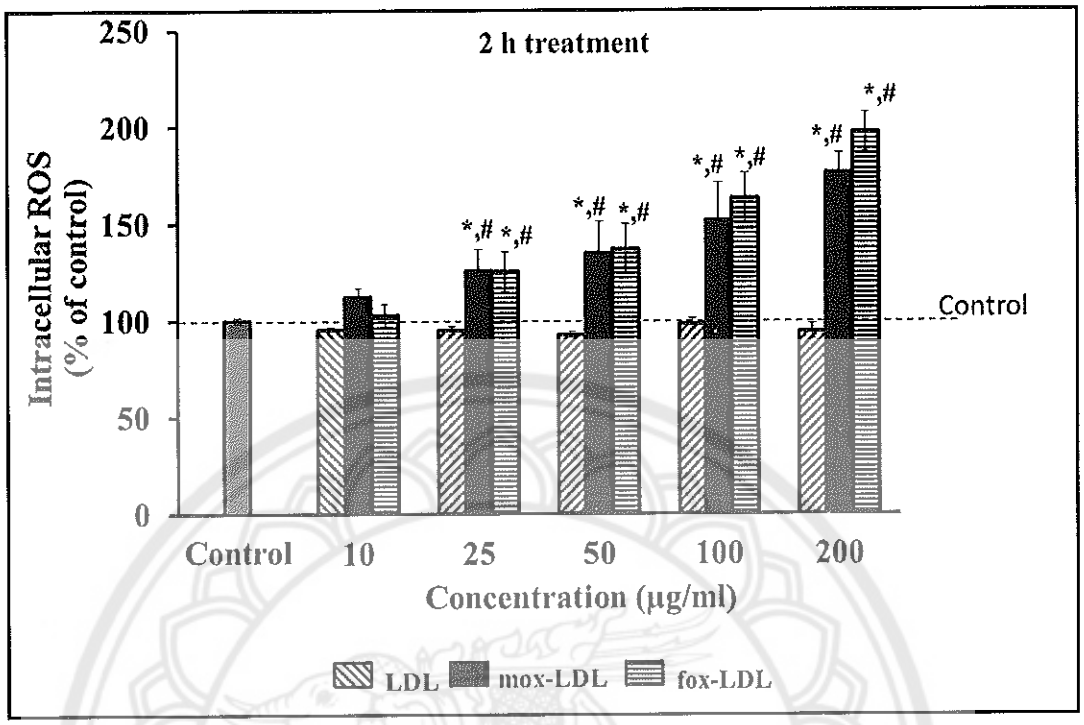



**Figure 13 Effect of LDL and oxidized LDL on intracellular ROS at 2 h treatment**

**Note:** SH-SY5Y neuroblastoma cells were treated with 10, 25, 50, 100 and 200 µg/ml of LDL preparations for 2 h. Fluorescence of DCF measured at 480 nm excitation and 530 nm emission was refered to intracellular ROS levels. Data present as mean ± SEM from at least five independent experimen ts. (* $p \leq$ 0.05 versus control or untreated cells, # $p \leq 0.05$ versus LDL)

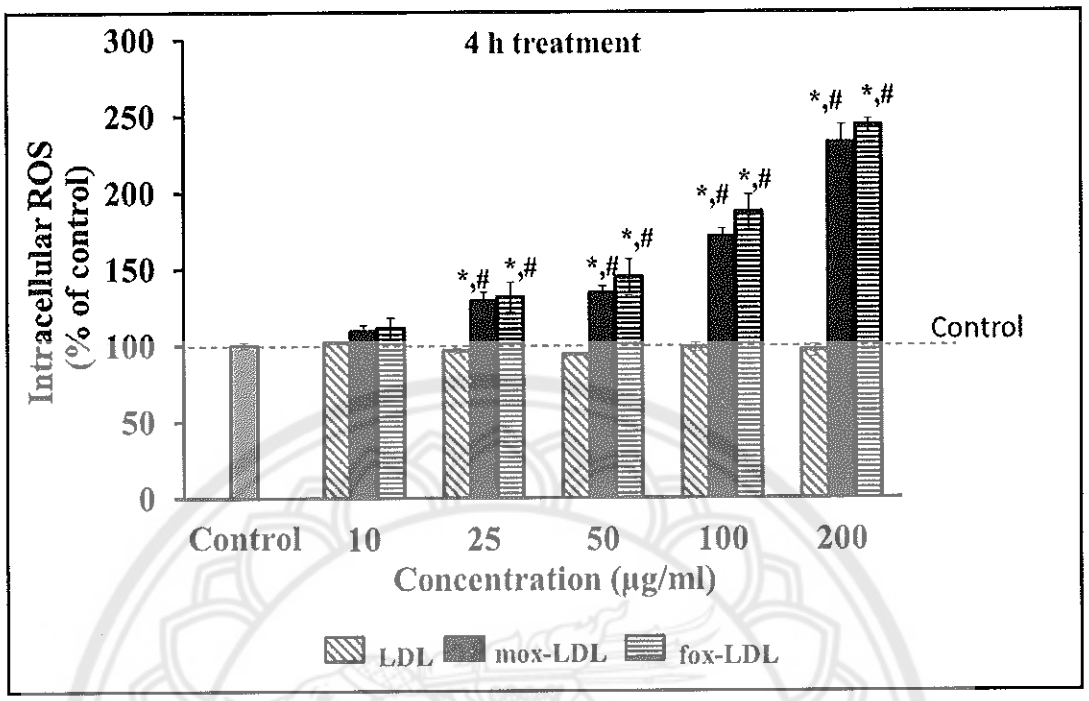



**Figure 14 Effect of LDL and oxidized LDL on intracellular ROS at 4 h treatment**

**Note:** SH-SY5Y neuroblastoma cells were treated with 10, 25, 50, 100 and 200 µg/ml of LDL preparations for 4 h. Fluorescence of DCF measured at 480 nm excitation and 530 nm emission was refered to intracellular ROS levels. Data present as mean ± SEM from at least five independent experimen ts. (* $p \leq$ 0.05 versus control or untreated cells, # $p \leq 0.05$ versus LDL)

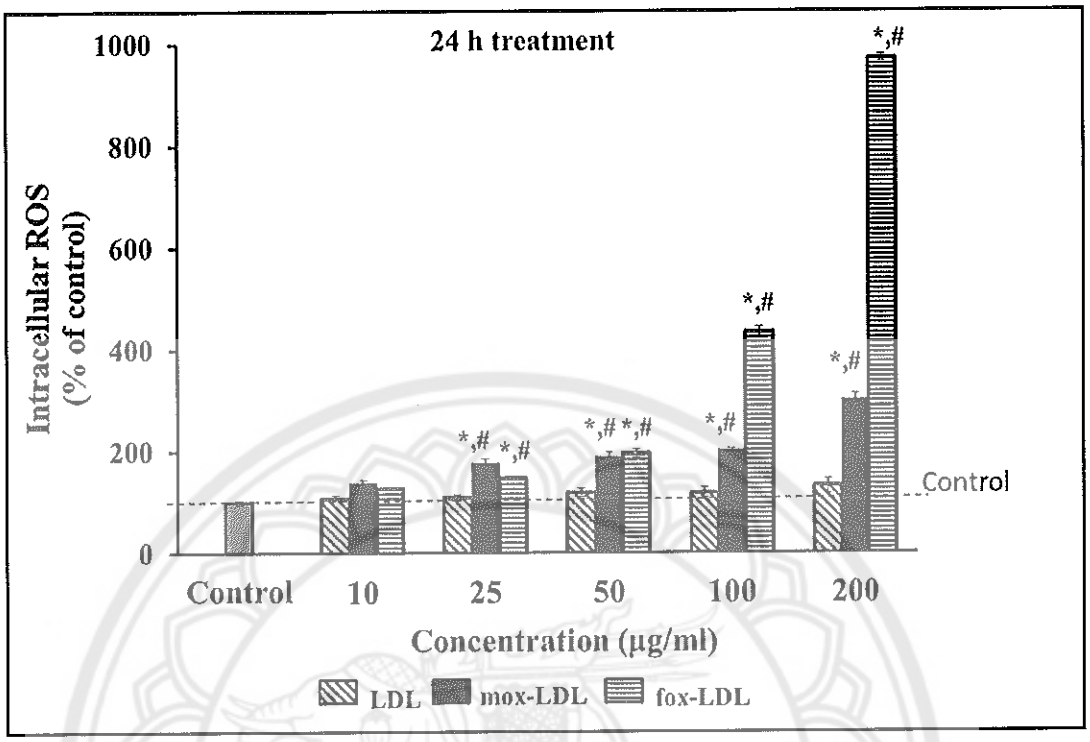



**Figure 15 Effect of LDL and oxidized LDL on intracellular ROS at 24 h treatment**

**Note:** SH-SY5Y neuroblastoma cells were treated with 10, 25, 50, 100 and 200 µg/ml of LDL preparations for 24 h. Fluorescence of DCF measured at 480 nm excitation and 530 nm emission was refered to intracellular ROS levels. Data present as mean ± SEM from at least five independent experimen ts. (* $p \leq 0.05$ versus control or untreated cells, # $p \leq 0.05$ versus LDL)

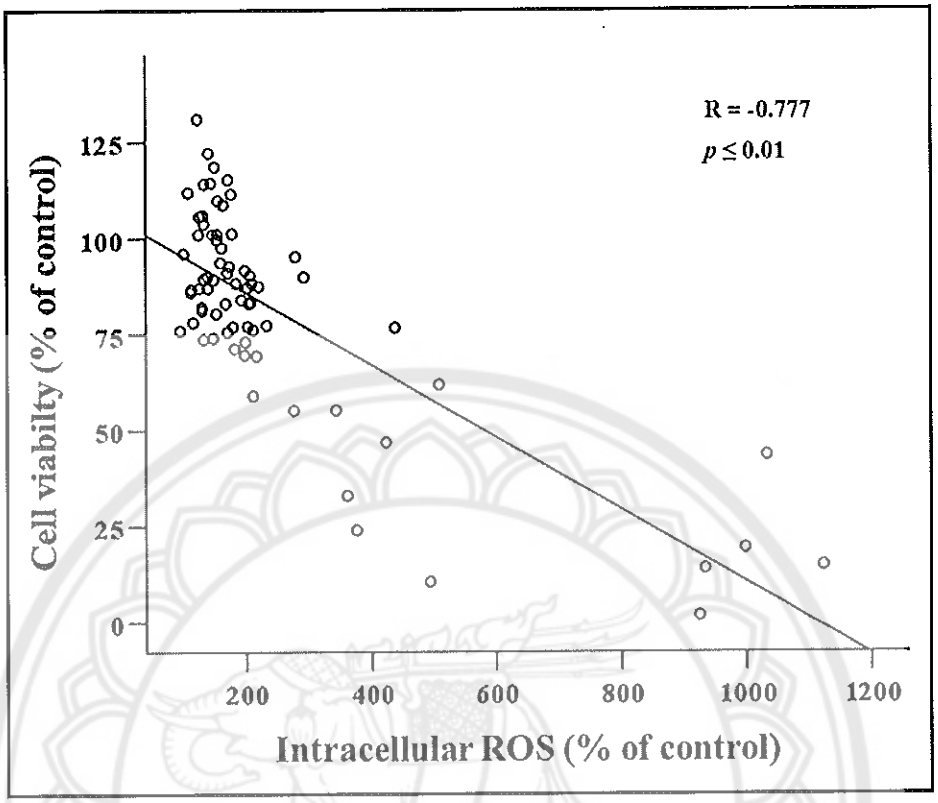



**Figure 16 Correlation between intracellular ROS and cell viability**

**Note:** Each data point is the result from individual treatment (cells treated with LDL and oxidized LDL for 24 h). Data were analyzes by Pearson's correlation

**3. Effect of LDL and oxidized LDL on type of cell death**

Caspase is the mediator in the signaling pathway of apoptosis, especially, caspase-3 as effector caspase. Necrotic cell death can be rapidly induced which is characterized by cell swelling, loss of membrane permeability and finally cell lysis [119]. Lactate dehydrogenase (LDH) is a soluble enzyme located in the cytosol and released into the surrounding culture medium upon cell membrane damage and indicated cell necrosis [56]. Thus, this study aimed to investigate whether oxidized LDL would induce apoptotic or necrotic cell death by measuring caspase 3 activity and LDH release, respectively.

LDH release in culture medium was assessed by LDH activity assay. After 24 h treatment with native LDL sample, cells showed no sign of cell membrane damage as LDH release was not detected (Figure 17). Mox- and fox-LDL at 100 and 200 μg/ml concentrations were toxic to neuronal cells as the increased LDH release was observed (Figure 17). The levels of LDH release induced by fox-LDL condition was higher than mox-LDL. It should be noted that expected concentration of $Cu^{2+}$ (1 μM) remaining in sample was tested and showed no effect on LDH release.

To determine apoptotic death, caspase-3 activity was measured in SH-SY5Y cells lysate after 24 h treatment with oxidized LDL at 50 and 100 μg/ml. The results showed that only 100 μg/ml of mox-LDL but not native LDL and fox-LDL increased caspase-3 activity in SH-SY5Y cells (Figure 18). It should be noted that fox-LDL at 200 μg/ml induced a large number of cell death. Thus, the amount of survival cells were not sufficiently to determine caspase-3 activity.

Our data suggest that degree of LDL oxidation differently affected type of cell death in which fox-LDL caused necrotic death whereas mox-LDL induced apoptosis. When LDL was highly oxidized lipid peroxidation products would be generated in large amount and might directly cause membrane damage leading to necrotic death. ROS have been reported damage lipid membrane in mammals [70] and breakage of lipid with formation reactive compounds can led to disrupt the permeability and fluidity of cell membrane and dramatically can alter the cell integrity [123]. Mox-LDL, lipoprotein particles were expected to be able to bind to oxidized LDL receptors and consequently lead to some changes in cellular signaling and finally to apoptotic death. Toll-like receptor activation was showed to a crucial role for

oxidized LDL induced apoptosis [23, 24]. When LDL is intensively oxidized, it may losses the ability to bind or activate receptor. Compositions and final products from lipid and protein oxidation in LDL complexes were reported to be dependent on oxidation condition [124] and this might reflex these consequence. Thus, receptor binding/activation of oxidized LDL seems to be important for apoptotic intracellular response in neurons.

Oxidized LDL have showed to increase the level of $Ca^{2+}$ and ROS in cultured cortical neurons and caused caspase activation [25]. Acrolein and HNE increased the level of active phosphorylation of c-jun that promote cell apoptosis in cultured neuron. HNE induced cellular oxidative stress, mediated apoptosis in PC12 and primary rat hippocampal neuron [119] and also impaired $Na^+$, $K^+$-ATPase activity in hippocampal neuron [125]. It is possibly that mox-LDL might induce intracellular ROS causing neuronal cell death through activation of c-jun. Lysophosphatidic acid in oxidized LDL can induce both neuronal apoptosis and necrosis in hippocampal neurons [126]. Moderate oxidative level have been showed to trigger cell apoptosis while higher oxidative levels may cause necrosis [72]. Thus, our data support that lower degree of LDL oxidation and lipid peroxidation products caused cell apoptotic death whereas higher degree of LDL oxidation promoted necrotic cell death.

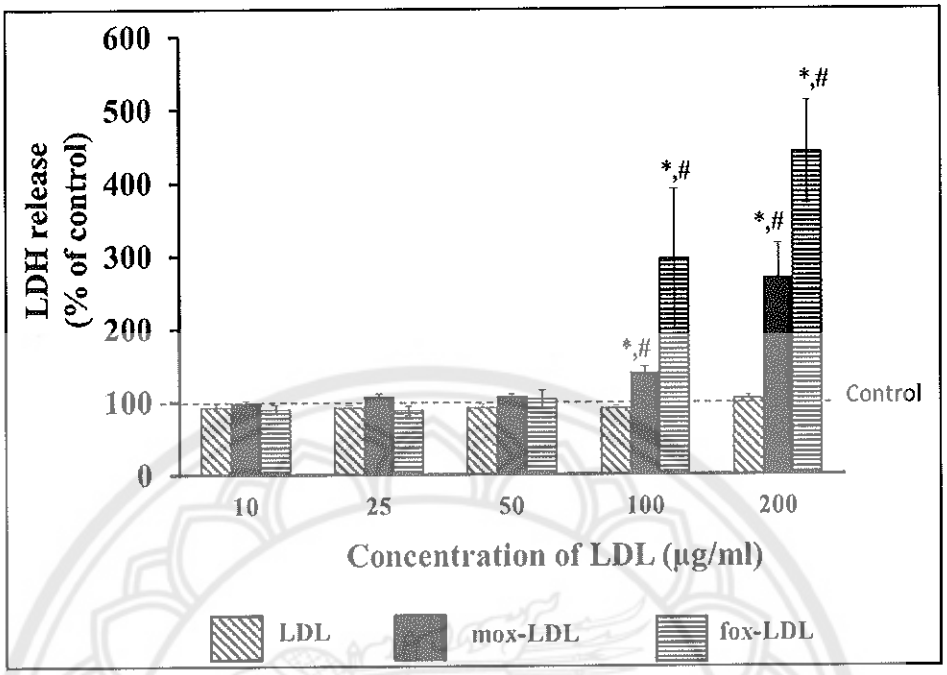



**Figure 17 Effect of LDL and oxidized LDL on cell membrane damage**

**Note:** SH-SY5Y neuroblastoma cells were treated with 10, 25, 50, 100 and 200 µg/ml of LDL preparations for 24 h. LDH released into culture medium was assessed by LDH assay and calculated as percent of control or untreated cells. Data present as mean±SEM from at least five independent experiments. (* $p \leq$ 0.05 versus control, # $p \leq 0.05$ versus LDL)

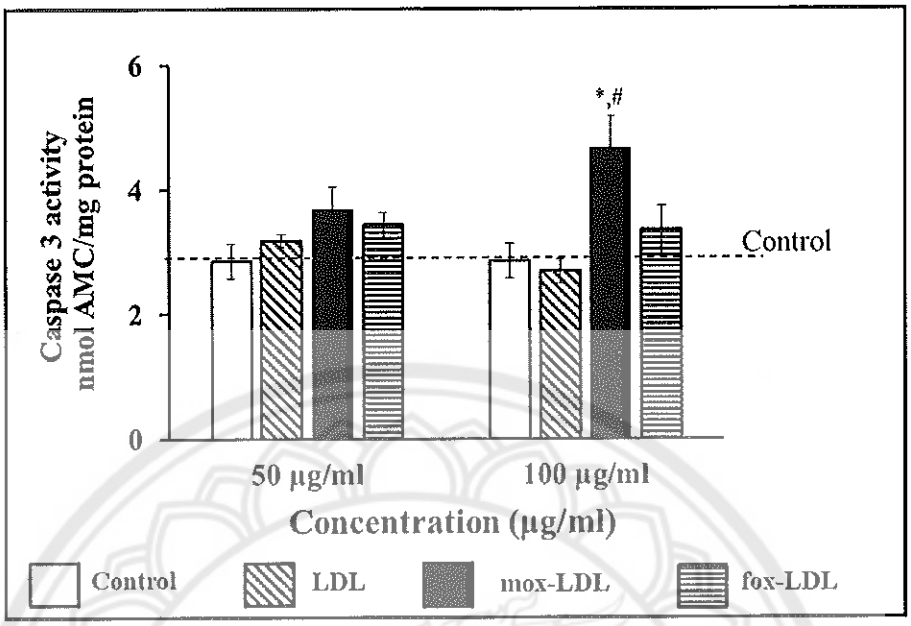



**Figure 18 Effect of LDL and oxidized LDL on apoptosis**

**Note:** SH-SY5Y neuroblastoma cells were treated with 50 and 100 µg/mL LDL preparations for 24 h. Cells were lysed and protein concentrations were determine by BCA assay kit. Caspase-3 activity in cell lysate were determined by Caspase-3 fluorimetric assay kit. Ac-DEVD-CHO is caspase-3 inhibitor. The data is presented as means±SEM from at least 4 different batches of cells. (* $p \leq 0.05$ versus control, # $p \leq 0.05$ versus LDL)

**4. Effect of LDL and oxidized LDL on cellular acetylcholinesterase (AChE)**

AChE is associated with the pathological process of AD as AChE inhibitors are used to restore ACh levels to improve cognitive functions [34]. In severe stage of AD, patients demonstrated a significant increase in AChE activity [127]. The activity of cellular AChE was previously showed to be enhanced by beta-amyloid peptides [128, 129], & tert-butylhydroperoxide [51], & these might be the consequence of oxidative stress. Thus, our study aimed to determine the effect of LDL and oxidized LDL on cellular AChE activity.

After treating differentiated SH-SY5Y cells with various oxidized LDL concentrations. Cellular AChE activity was slightly increased at 1 and 2 h after treatment with oxidized LDL all tested concentrations but native LDL did not have this effect (Figure 19, 20). At 4 and 24 h treatment with oxidized LDL, cellular AChE activities were obviously enhanced in dose dependent patterns (Figure 21, 22). Cells treated with native LDL (200 µg/ml for 24 h) showed slightly enhanced AChE activity (approximately 40%) but statistically significance was not observed (Figure 22). It should be noted that cellular AChE activities herein were evaluated based on the equivalent number of survived cells after each treatment. Further data analysis demonstrated positive correlation between intracellular ROS levels and AChE activities (Figure 23). These data indicate that the activity of AChE in neuroblastoma cells could be enhanced by oxidized LDL and this phenomenon might be on oxidative stress.

The increases in AChE activity could be the result of the induction of AChE protein level in neurons. Thus, further experiment was aimed to test whether oxidized LDL affected AChE protein expression. Because high concentration of oxidized LDL killed large number of cells, so that subtoxic concentration of oxidized LDL (50 µg/mL) was chosen for this experiment. Cells were treated with all LDL samples (50 µg/ml) for 24 h and the levels of AChE protein was determined by western blot. Cellular AChE activity was also determined in cell lysate. The results showed that all LDL samples did not change the AChE levels (Figure 24, A) but both oxidized LDL increased cellular AChE activity (Figure 24, B). These results suggest that oxidized LDL enhances enzymatic activity of AChE, possibly directly through cellular oxidative mechanism but not involved protein synthesis.

In our data, AChE activity in neuron showed positive correlation with intracellular ROS (Figure 23). Previously, $H_2O_2$ have showed to induce AChE activity in PC12 cells and glutathione attenuated this increment of AChE activity [130]. This elevated AChE activity was thought to be consequent from ROS and was observed after the degradation of Akt and the release of cytochrome c from mitochondria into cytosol and caspase activation [130]. The increase in caspase -3 activity following increased ROS was associated with AChE activity, indicating a correlation between AChE and neuronal apoptosis. Oxidation of LDL might disrupt the function of apolipoprotein B100 that recognize and bind to LDL receptor. The alteration of the surface charge on the apolipoprotein B100 was occurred after oxidation of LDL [110]. The electrophoretic mobility of ApoB100 were 0.46 and 1.46 after oxidation for 45 min and 24 h respectively, while native LDL was 0.30, relative to albumin [110]. Thus, mox-LDL possibly could bind to the LOX-1 receptor resulted in increased caspase-3 activity and AChE activity during apoptosis. Fox-LDL could losses the ability the bind to receptor. This study indicate that the oxidized LDL-receptor binding seem to be important for cellular response in neuron. However, high concentration of lipid peroxidation products might be directly generate the free radicals resulting increased AChE activity and apotosis in neuron, independently from receptor binding pathway. Figure 25 demonstrated the AChE activity, the release of LDH, & caspase-3 activity after treatment cells with 100 µg/ml of all LDL preparations. These results showed that the increase in AChE was not associated with apoptosis but seem to be correlated with necrosis. Our data indicate that different degrees of LDL oxidation might mediate neuronal degeneration via different pathways.

There is no direct evidence demonstrating how AChE correlated with necrosis in neuron cells. Based on previous study, purified AChE changes the morphology and increased the release of LDH release in neuronal (Neuro-2) and glia-like (B-12) but these toxic effects of AChE were independent of its catalytic active site [131]. The inhibition of AChE by BW 248C51, an inhibitior of AChE, decreased the neurite outgrowth indicating AChE can regulate neurite outgrowth and have a non-cholinergic action [131]. Readthrough acetylcholinesterase (AChE-R), a splice variant of AChE, was showed to have a non-cholinergic function and its expression is increased upon oxidative stress [132]. AChE-R regulated necrosis in human ovarian

cells via the receptor-interacting protein kinase 1(RIPK-1) [132]. TNF-induced ROS production also promoted necrosis by the regulation of RIP3 in fibroblast NIH 3T3 cell. [133]. It is possible that oxidized LDL-induced intracellular ROS was associated increased AChE activity resulting necrosis by RIP pathway.

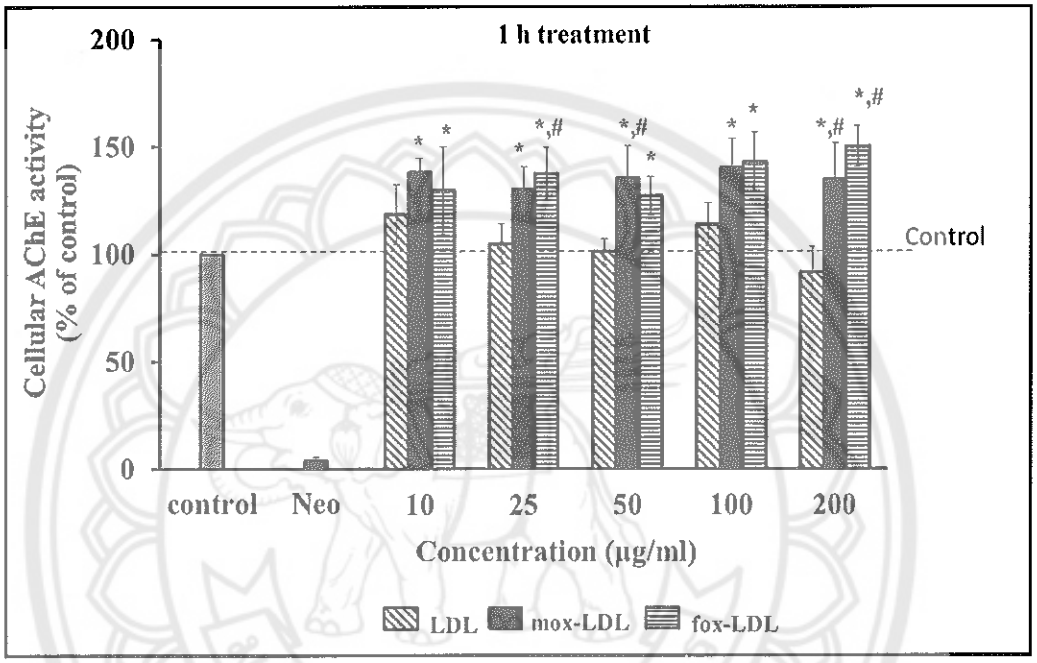



**Figure 19 Effect of LDL and oxidized LDL on cellular AChE of SH-SY5Ycells after 1 h treatment**

**Note:** SH-SY5Y neuroblastoma cells were treated with 10, 25, 50, 100 and 200 µg/mL LDL preparations for 1 h. AChE activity was measured by Ellman's method and expressed as a percent of control or untreated cells. Data present as mean±SEM from at least five independent experiments (* $p \leq 0.05$ versus control, # $p \leq 0.05$ versus LDL, Neo = neostigmine, an AChE inhibitor)

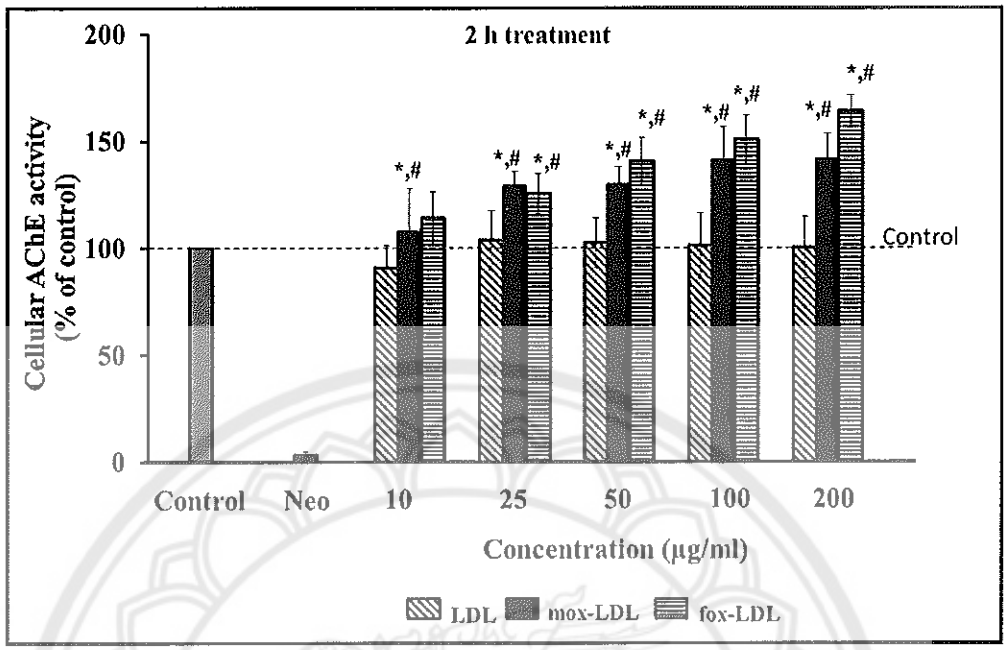



**Figure 20 Effect of LDL and oxidized LDL on cellular AChE of SH-SY5Ycells after 2 h treatment**

**Note:** SH-SY5Y neuroblastoma cells were treated with 10, 25, 50, 100 and 200 µg/mL LDL preparations for 2 h. AChE activity was measured by Ellman's method and expressed as a percent of control or untreated cells. Data present as mean±SEM from at least five independent experiments (* $p \leq 0.05$ versus control, # $p \leq 0.05$ versus LDL, Neo = neostigmine, an AChE inhibitor)

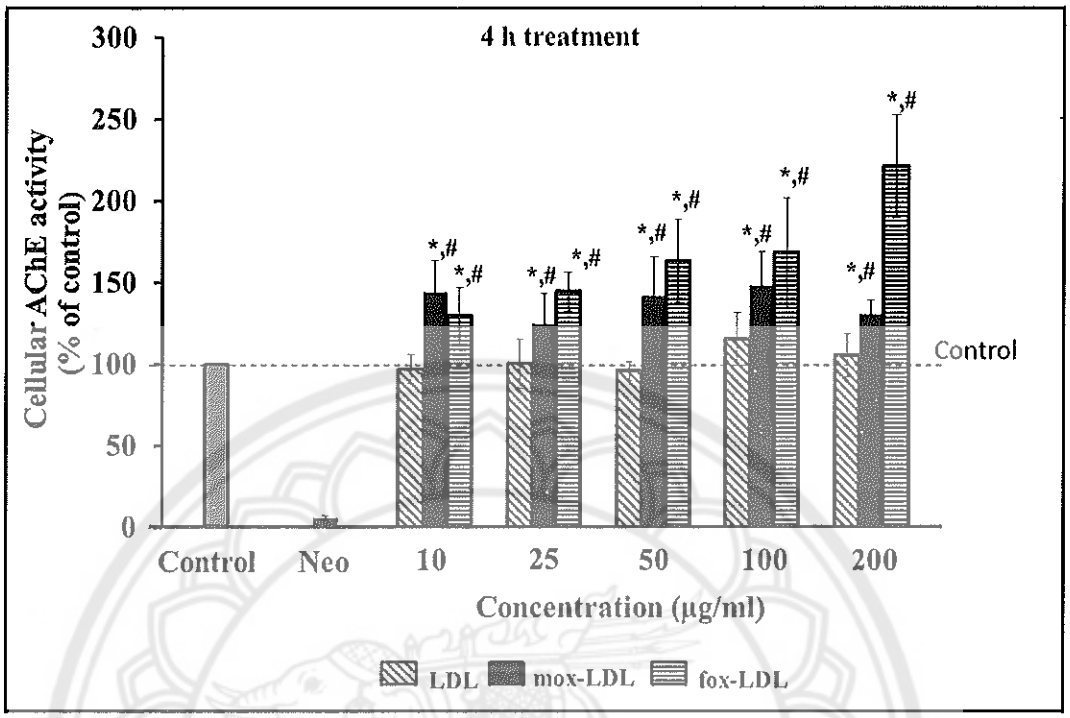



**Figure 21 Effect of LDL and oxidized LDL on cellular AChE of SH-SY5Ycells after 4 h treatment**

**Note:** SH-SY5Y neuroblastoma cells were treated with 10, 25, 50, 100 and 200 µg/mL LDL preparations for 4 h. AChE activity was measured by Ellman's method and expressed as a percent of control or untreated cells. Data present as mean±SEM from at least five independent experiments (* $p \leq 0.05$ versus control, # $p \leq 0.05$ versus LDL, Neo = neostigmine, an AChE inhibitor)

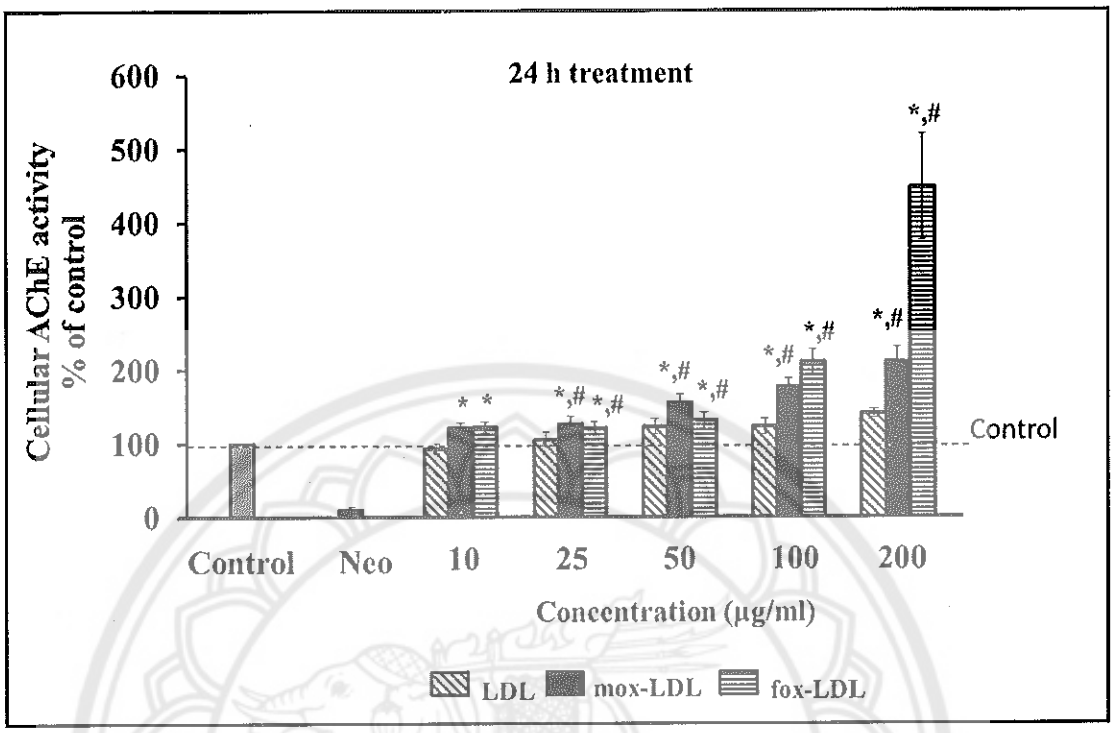



**Figure 22 Effect of LDL and oxidized LDL on cellular AChE of SH-SY5Ycells after 24 h treatment**

**Note:** SH-SY5Y neuroblastoma cells were treated with 10, 25, 50, 100 and 200 µg/mL LDL preparations for 24 h. AChE activity was measured by Ellman's method and expressed as a percent of control or untreated cells. Data present as mean±SEM from at least five independent experiments (* $p \leq 0.05$ versus control, # $p \leq 0.05$ versus LDL, Neo = neostigmine, an AChE inhibitor)

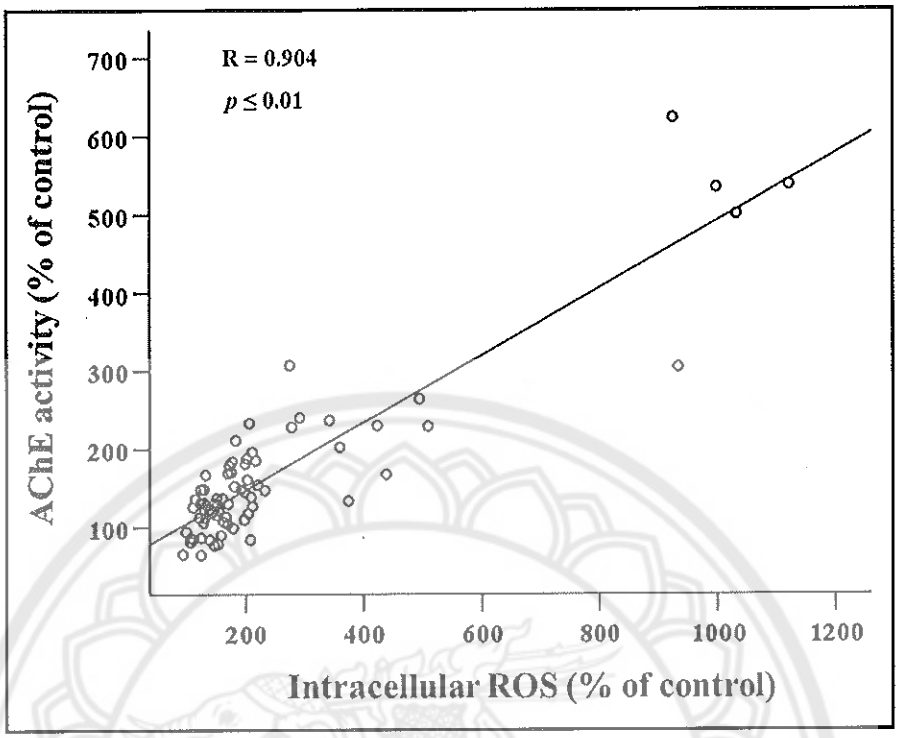



**Figure 23 Correlation between intracellular ROS and AChE activity**

**Note:** Each spot is the result from individual treatment of cells treated with LDL and oxidized LDL for 24 h. Data were analyzes by Pearson's correlation

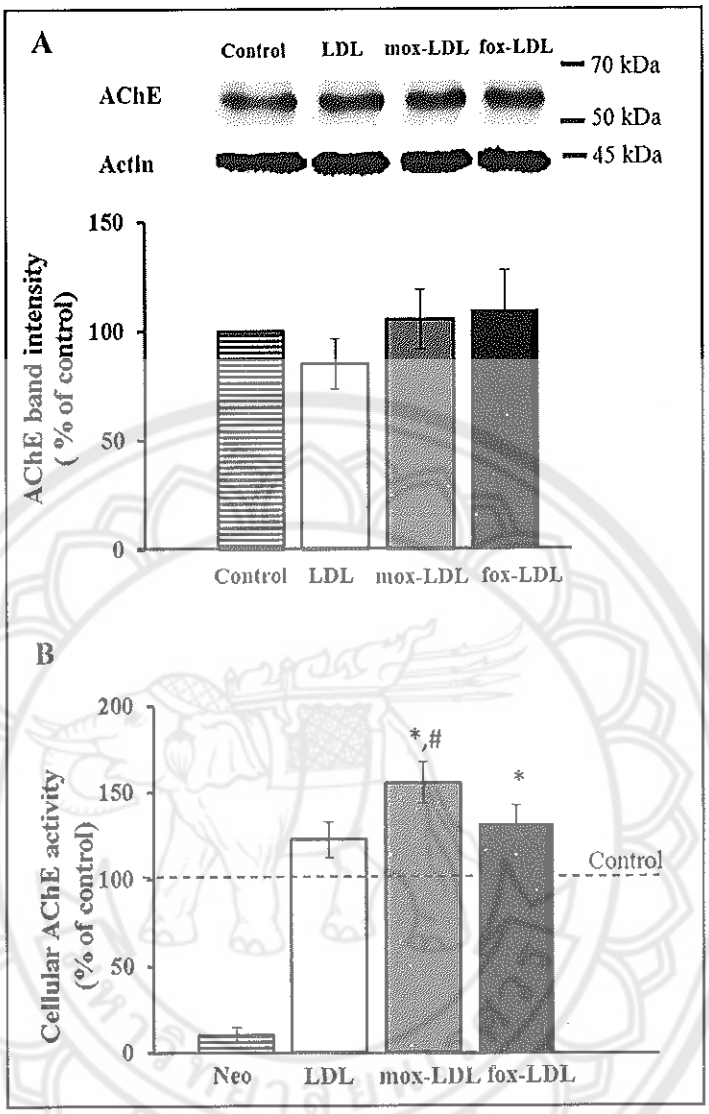



**Figure 24 Effect of LDL and oxidized LDL on AChE activity and expression**

**Note:** (A) After treating cells with 50 μg/mL of LDL for 24 h, cells were lyzed and AChE activity measured. (B) Cell lysates from separated experiments were prepared and the AChE level was determined using anti-AChE (ab 31276; 1:1000) by Western blot. The data is presented as means±SEM from at least 4 different batches of cells. (* $p \leq 0.05$, versus control)

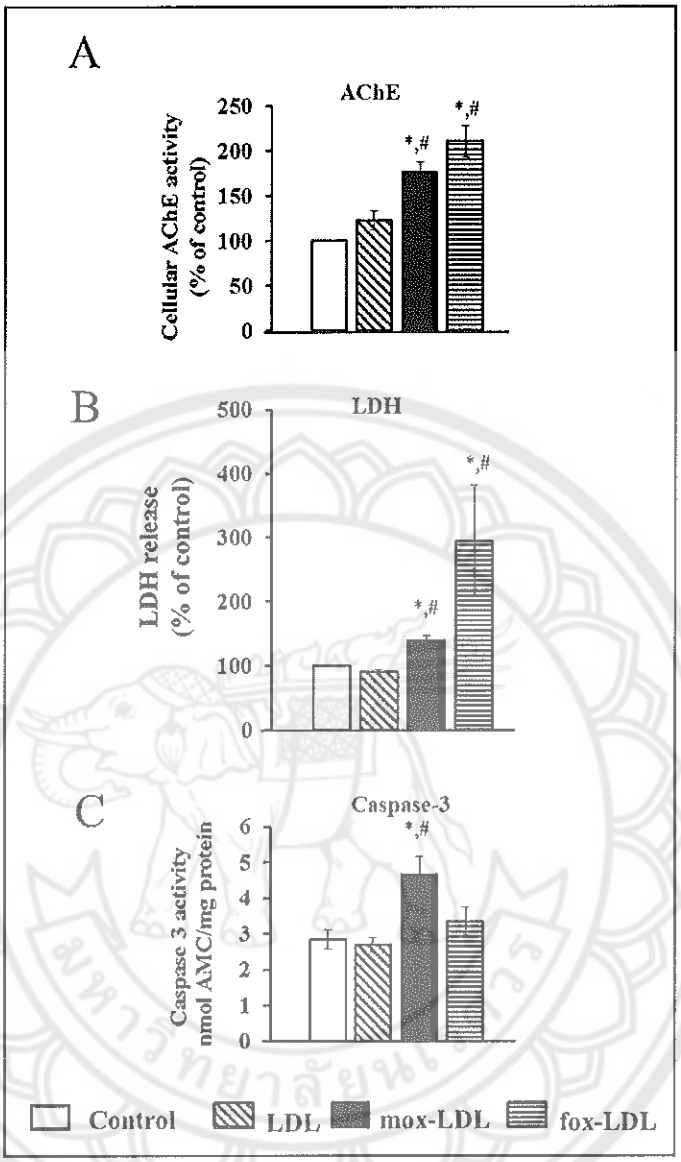



**Figure 25 Comparison of AChE activity, LDH release, & Caspase-3 activity**

**Note:** SH-SY5Y cells were treated with 100 μg/mL of LDL preparations for 24 h. The AChE activity was measured by Ellman's method (A). Cell membrane damage was assessed by LDH into culture medium (B) and Caspase-3 activity in cell lysate was determined by Caspase 3 assay kit (C). The data is presented as means±SEM from at least 4 different batches of cells. (* $p \leq 0.05$, versus control, # $p \leq 0.05$ versus LDL)

**5. Effect of LDL and oxidized LDL on APP metabolism**

The main pathology of AD is the deposition of Aβ in the brain. It is released from large amyloid precursor protein (APP) through proteolytical cleavage by secretase enzyme including α, β and γ-secretase (Figure 29). The β-secretase claves APP at the beginning of the sequence of the Aβ and release soluble APPβ (sAPPβ) fragment into extracellular. This process is called the amyloidogenic pathway that leads to Aβ accumulation and neurotoxicity [45]. In contrast, the α-secretase cleaves APP at middle of large soluble APP and then secretes soluble APPα (sAPPα) that does not accumulate to amyloid plaque [45]. This process is the non-amyloidogenic pathway. This study aimed to investigate the effect of LDL and oxidized LDL on APP processing.

The release of sAPPα and sAPPβ was measured in culture medium after incubating cells with all three LDL preparations. Soluble APP presenting in medium was expected to be the cleavage product from precursor membrane bound APP of normal adherence cells. Therefore, membrane damage from injured neuronal cells might interfere level of sAPP in culture medium. Thus, subtoxic concentration of oxidized LDL (50 μg/mL) was chosen for this set of the experiments. The amount of released sAPP in culture medium was concentrated and determined by immunoblotting. Because the amount of proteins released from cells to medium of each treatment was expected to be different, equivalent volume instead of equivalent total protein was used as loading control for SDS-PAGE.

After treating differentiated SH-SY5Y cells with 50 μg/mL of LDL for 24 h, medium was collected and showed the significant increase in the level of total APP compared to the control (Figure 26, A). Both forms of the oxidized LDL did not affect the level of APP in the medium. Because cultured cells could undergo unintentionally detached from the plate surface into medium, therefore level of actin from each medium preparation was also measured and used to normalize intensity of all sAPP bands. It should be noted that all antibodies used in this study were tested and showed no interaction with proteins in LDL and oxidized-LDL particles (Figure 27). APP was manifested as the typical double bands of immature APP695 and mature APP751/770 [43] appeared on the blot between 100-140 kDa and the intensities of upper and lower bands were correlated. The result showed that the secretion of total APP was increased

almost two folds by LDL but not affected by oxidized LDL (Figure 26 A). This increment was not the result of the increase in cellular expression of APP since total APP levels in cell lysates from all treatments were similar (Figure 28). These results suggest that LDL mediate APP metabolism in neuronal cells which then leads to the release of soluble APP.

To clarify types of released APP induced by LDL, specific antibodies against sAPP-α and sAPP-β were used in the immunoblotting (Figure 29). The result showed that soluble APP found in culture medium following native LDL treatment was mainly sAPP-β and partially sAPP-α (Figure 30, 31). Both forms of oxidized-LDL had no significant impact on sAPP-β and sAPP-α levels. These results suggest that LDL induces APP metabolism to the amyloidogenic rather than non-amyloidogenic pathway.

Beta-site APP cleaving enzyme 1 (BACE-1) or β-secretase is single domain integral protein with its active site located on the ectoplasmic side of the membrane. It is also detected within the TGN, endosomal compartment [47], lysosomal and ER [134]. BACE-1 activity is believed to be the main factor to catalyze and produced the amount of Aβ [42]. It is the first enzyme to cleavage the APP in amyloidogenic pathway. Cellular stress has been reported to enhance the BACE-1 expression and showed that BACE-1 level also increased in response to amyloid plaque [135]. Further experiment, cells treated with all LDL samples were determined for β-secretase activity and data showed no change with all LDL samples treatments (Figure 32).

In previous study, primary cerebral cortical neurons treated with LDL showed that LDL induced the increased Aβ production and enhanced BACE-1 activity in accompany with increased accumulation of APP and BACE-1 in endolysosomes [12]. Endolysosome mechanism was thought to be a major effect of LDL on intraneuronal deposition of Aβ [136]. From our data, native LDL induced the release of sAPPβ whereas its oxidized form did not change the level of released sAPP. Although, the BACE-1 activity from our study did not change in native LDL treatment but LDL on BACE-1 activity from previous reports are inconsistent. Hui and colleagues showed that increased LDL cholesterol accumulation was hypothesized to reduce degradation of BACE-1 by increasing endolysosome pH resulting in increased

BACE-1 activity after 3 days exposure [12]. Later study indicated that BACE-1 activity was greatly increased in 2 h and much less activity was observed at 16 h after LDL treatment and these phenomena occurred in accordance with lipid raft formation [16]. Loss of BACE-1 activity at 16 h was thought to be the result of internalization of lipid raft and associated protein into endolysosome to undergo degradations [16]. It is possible that the amount of accumulated LDL cholesterol in endolysosome indicates BACE-1 activity. Previously, cholesterol was able to directly enhance BACE-1 activity as shown in the *in vitro* assay [137]. The activity of BACE-1 in AD brain was found to be more sensitive to cholesterol (40 μM) than that of normal brain (80 μM) [137]. This finding supports the importance of cholesterol concentration on BACE-1 activity. In our study, BACE-1 was measured at 24 h treatment with subtoxic concentration of LDL where cholesterol accumulation possible might not be sufficient to reduce degradation or enhance activity of BACE-1, so that increased BACE-1 activity was not observed.

In addition to LDL, oxidized LDL was reported to promote Aβ production and increase BACE-1 activity [16]. Lipid peroxidation products was also reported to increase the Aβ productions and BACE-1 activity [16]. However, oxidized LDL from our study on the other hand neither induced the release of sAPPβ nor promoted BACE-1 activity. Thus, the neurodegenerative activity of oxidized LDL was potentially due to other pathways. Neurotoxicity effect of oxidized LDL were previously showed to be associated with several cellular mechanisms including increased intracellular ROS and $Ca^{2+}$, depleted glutathione level, & increased caspase activity [23, 25, 120].

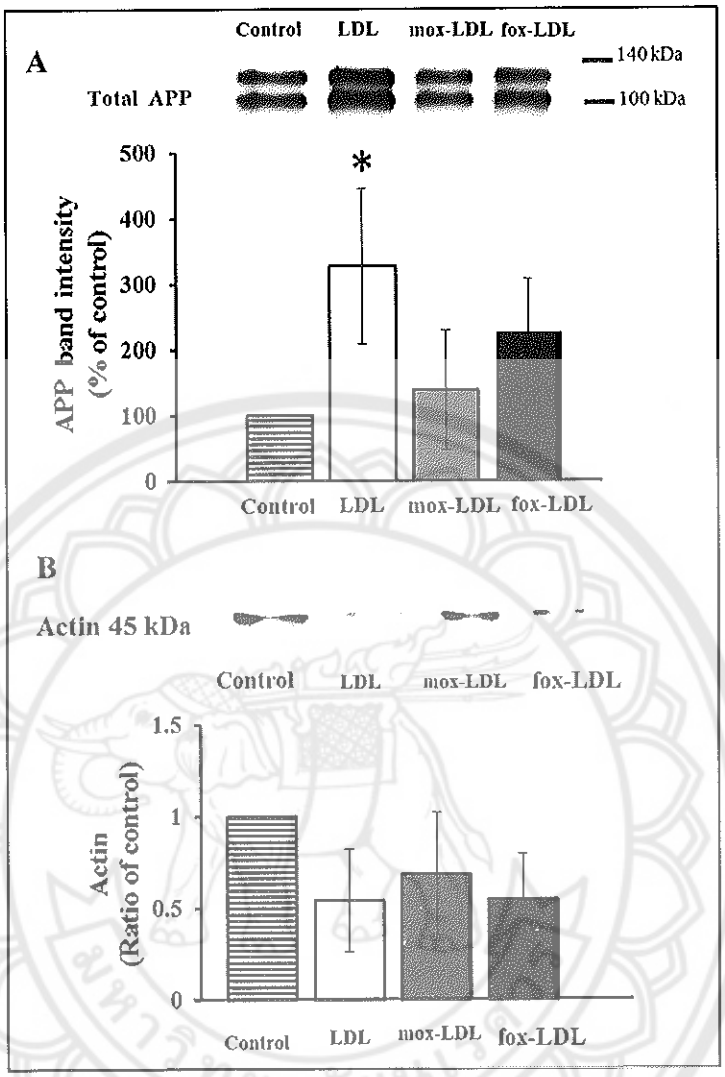



**Figure 26 The effect of LDL and oxidized LDL on the release of APP**

**Note:** SH-SY5Y cells were treated with LDL and oxidized LDL at 50 μg/mL of each LDL preparation for 24 h. Soluble APP secreted into cultured medium was collected and analyzed by western blot. Actin was determined in culture medium as well. The data is presented as means ± SEM from at least 5 different batches of cells. (* $p \leq 0.05$, versus control)

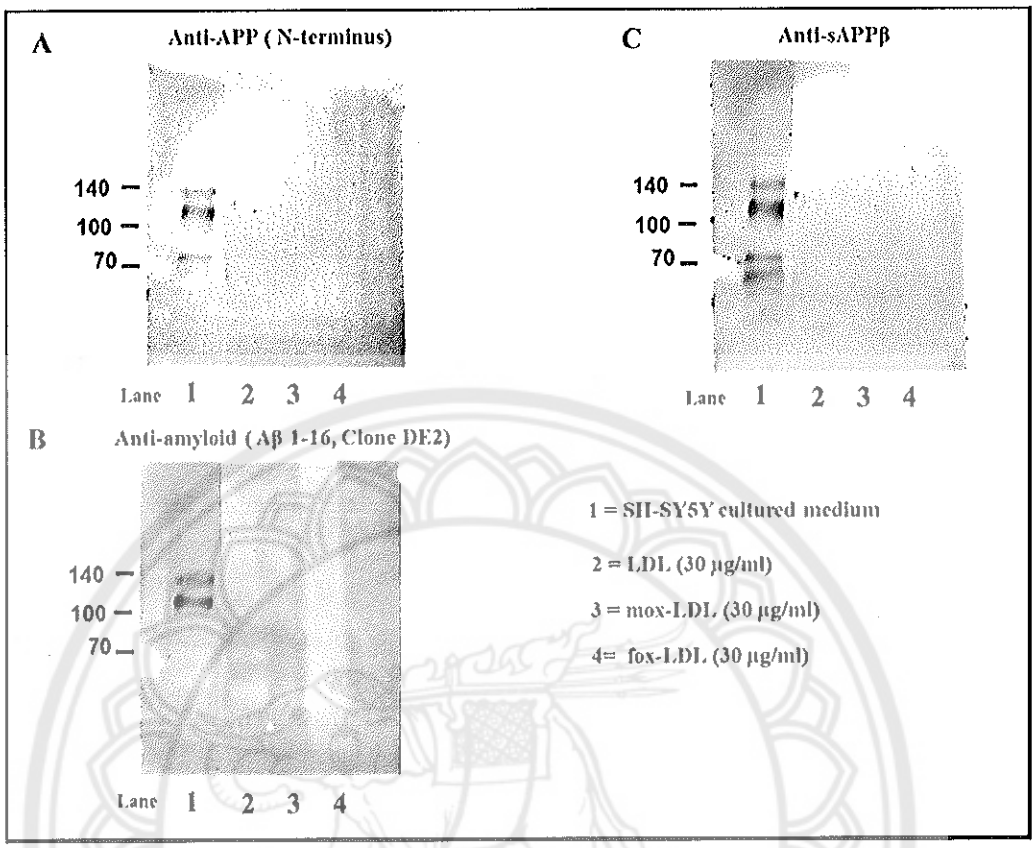



**Figure 27 The interaction of proteins in LDL and oxidized-LDL on all antibodies**

**Note:** SH-SY5Y concentrated media ( 30 µl, lane 1), LDL (30 µg/ml, Lane 2), mox-LDL (30 µg/ml, lane 3) and fox-LDL (30 µg/ml, lane 4) were run into SDS-PAGE and transferred to PVDF membrane. The bands were analyzed by western blot using specific antibodies that shown in Figure 29

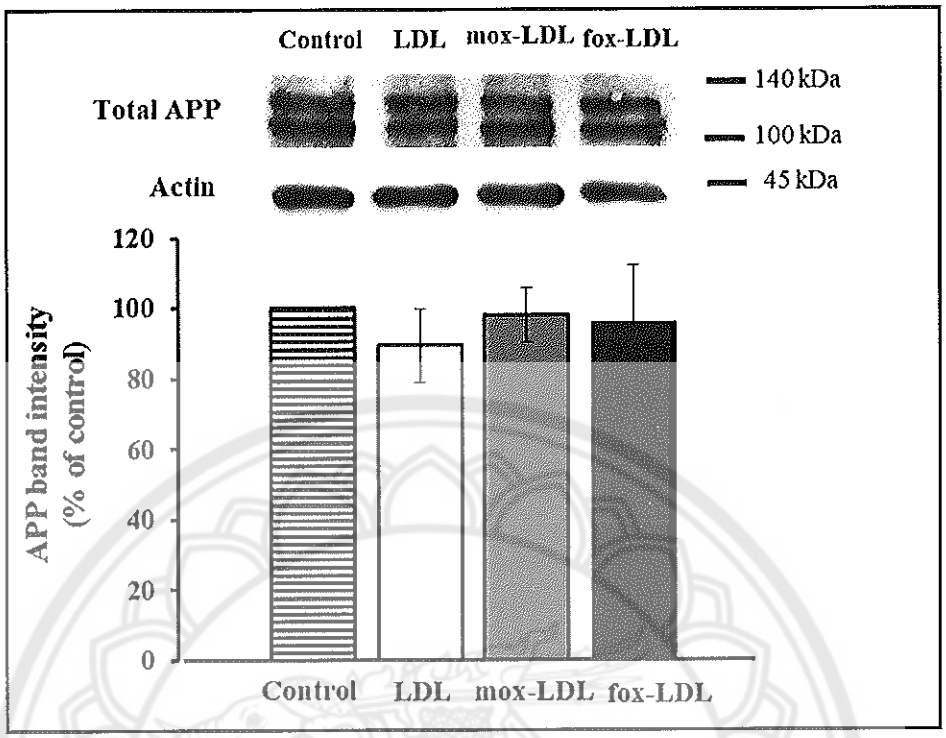



**Figure 28 APP expression in cell lysate**

**Note:** SH-SY5Y cells were treated with LDL and oxidized LDL at 50 µg/ml for 24 h. Cells were lysed and protein concentration was determined by BCA assay. APP expression was analyzed using anti-APP (N-terminus) by western blot. The data is presented as means ± SEM from at least 5 different batches of cells. (* $p \leq 0.05$, versus control)

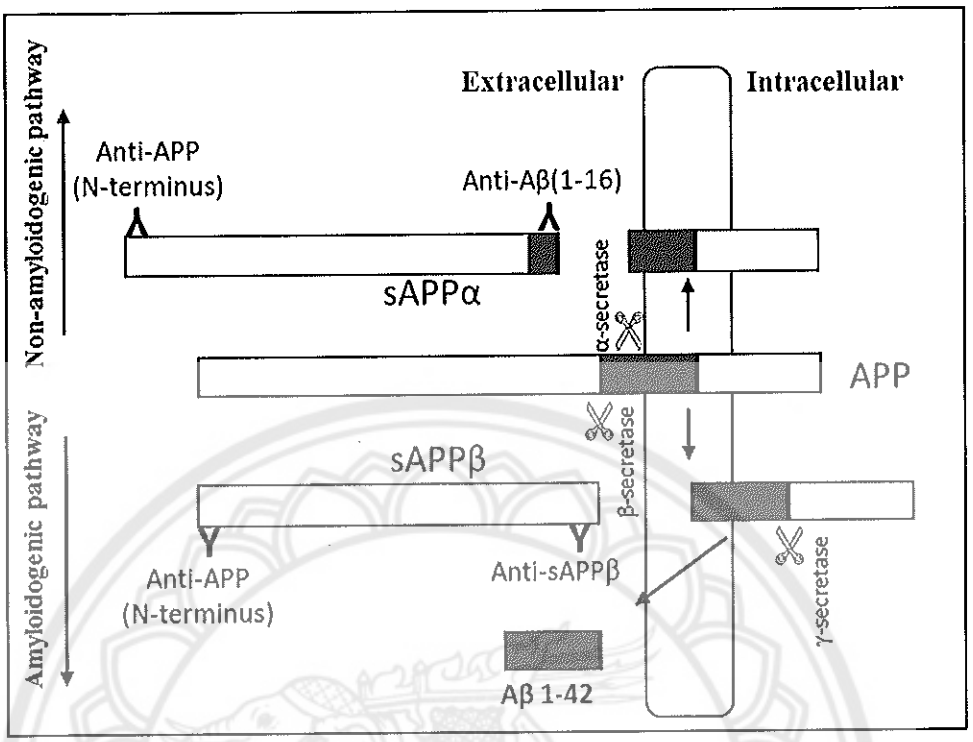



**Figure 29 Schematic of APP processing and specification of antibodies**

**Note:** The anti-APP recognizes APP near the N-terminus. The anti-amyloid (Aβ 1-16, Clone DE2) recognizes the amyloid beta 1-16 residue in the sAPPα which is clevaged by α-secretase. The anti-sAPPβ can detect sAPPβ which is cleaved by β-secretase and does not cross-reacts with sAPPα and full length APP

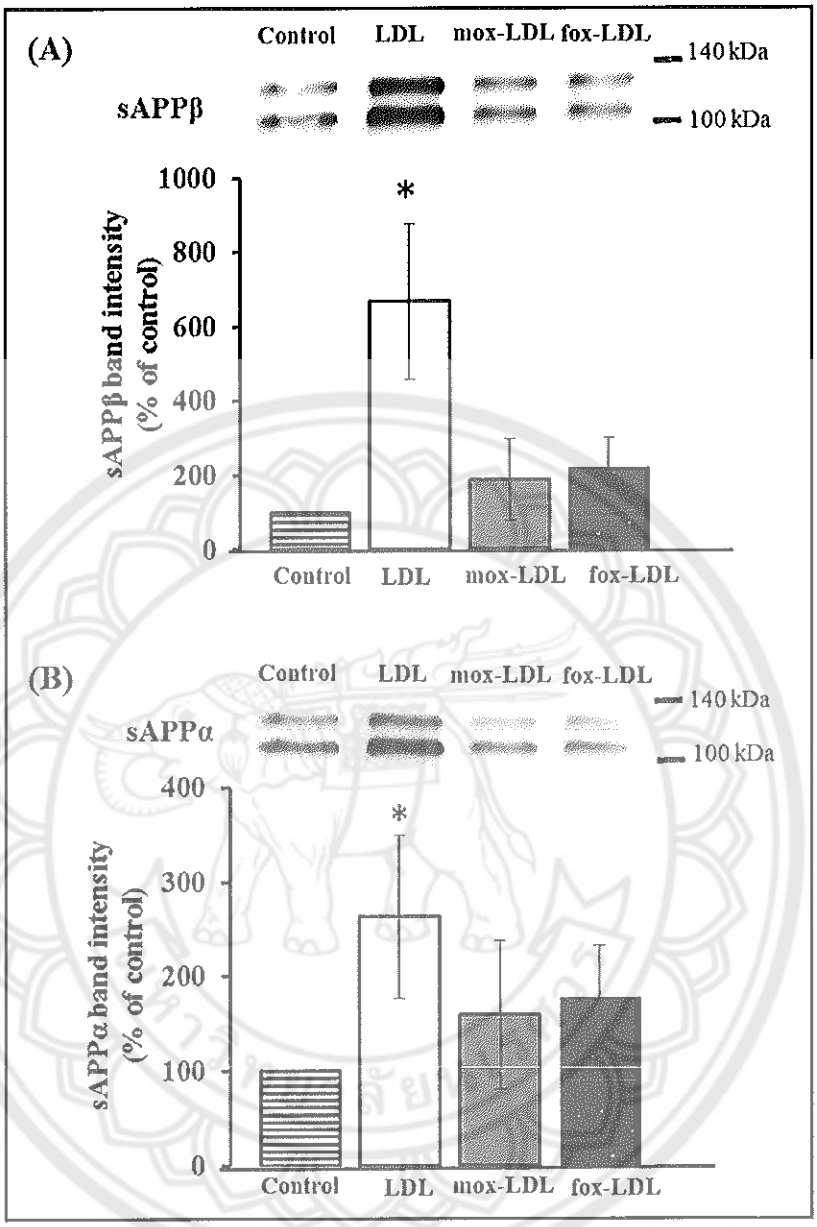



**Figure 30 Levels of sAPPα and sAPPβ release into culture medium**

**Note:** Levels of sAPPα and sAPPβ released from cells to culture medium after treating with LDL and oxidized LDL. Cells were treated with 50 μg/mL of each LDL preparation for 24 h. Level of sAPPβ and sAPPα was detected by western blotting using anti-sAPPβ wild type (A) and anti-Aβ (1-16) (B), respectively. The data is presented as means±SEM from at least 5 different batches of cells. (* $p \leq 0.05$, versus control)

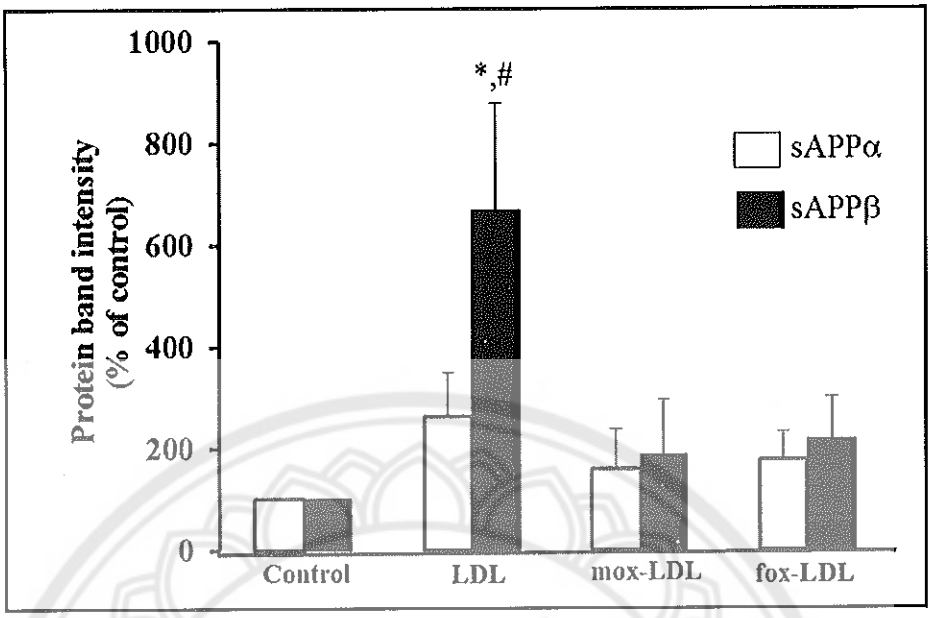



**Figure 31 Comparison of sAPPα and sAPPβ release into culture medium**

**Note:** The data is presented as means±SEM from at least 5 different batches of cells. (* $p \leq 0.05$, versus control; # $p \leq 0.05$ versus LDL)

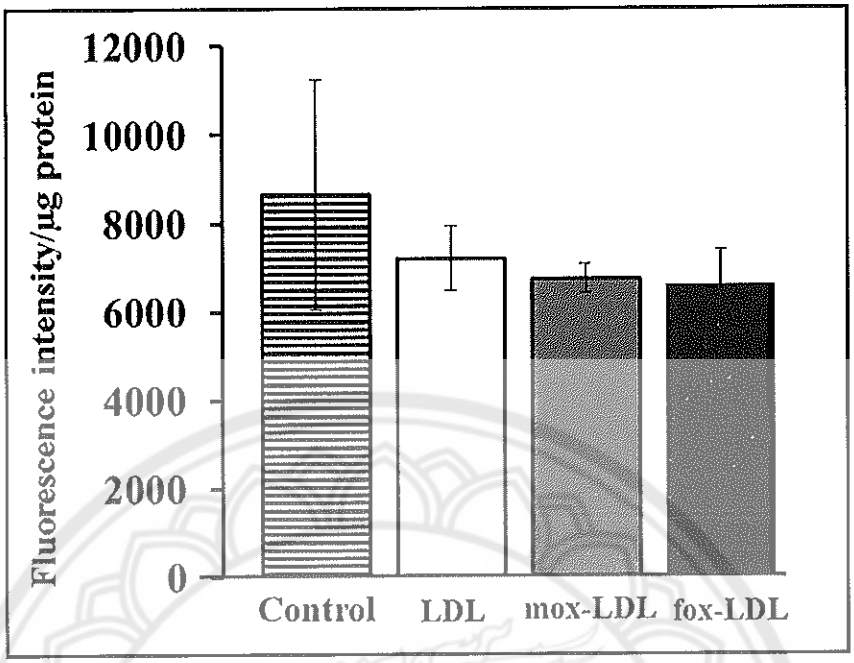



**Figure 32 Effect of LDL and oxidized LDL on β–secretase activity**

**Note:** SH-SY5Y neuroblastoma cells were treated with 50 µg/mL of each LDL preparation for 24 h. Cell lysates were prepared and measured for β–secretase activity. The data is presented as means±SEM from at least 4 different batches of cells

### 6. Effect of LDL and oxidized LDL on CREB signaling

CREB, a nuclear transcription factor, can be activated by phosphorylation at Serine-133 through various membrane receptors and intracellular pathways [61]. CREB phosphorylation is believed to be responsible as an activation of gene expression such as brain-derived neurotrophic factor (BDNF) [61]. These genes are important in neuroplasticity and cognitive function that required for learning and memory. Impaired CREB phosphorylation has been found to involve with pathogenesis of neurodegenerative disorder such as AD [67, 68]. It has been reported that oxidative stress decreased the level of total CREB and phosphorylated CREB (pCREB) in primary hippocampus neuron and neuronal cell line [68]. Thus, further our experiment aimed to investigate the effect of oxidized LDL on CREB signaling.

The phosphorylated CREB (pCREB) is an active form of CREB which is rapidly phosphorylated at serine 133 by several kinases and subsequently enters the nucleus and activates intracellular pathways to induce transcription of genes such as BDNF that contributes the survival, growth and function of hippocampus neuron [61]. From previous study, 10 μg/ml of LDL and oxidized LDL have been transiently increased pCREB at 15 min in vascular smooth muscle cell (VSMC) [138]. Thus, at subtoxic concentration of oxidized LDL (10 μg/ml) was chosen for this set of the experiment. SH-SY5Y cells were treated with all three LDL samples for 15 and 60 min. Nuclear extraction protein was prepared by nuclear extraction kit and pCREB and CREB protein level were determined by immunoblotting. The results showed that the level of CREB in the nuclear extract was transiently increased at 15 min after treating with LDL and reduced at 60 min (Figure 33). Mox-LDL also increased nuclear CREB level at 15 min and slightly reduced to normal at 60 min. Level of CREB in the nuclear was reduced by fox-LDL at both time points. This data showed that nuclear translocation of CREB can be transiently mediated by LDL and mox-LDL, but not fox-LDL.

The level of pCREB in nuclear extract protein was also determined by immunoblotting to evaluate the activation of CREB. The results showed that the level of pCREB were correlated with CREB level (Figure 34). The level of total CREB in cell lysate was also determined after treatment with native LDL and oxidized LDL for 24 h. The results showed that all LDL preparations did not change the CREB

expression (Figure 35). This data suggest that native LDL and oxidized LDL differently affect CREB phosphorylation in neuronal cells.

Our data demonstrated that native LDL transiently activated CREB in neuroblastoma cells. In vascular smooth muscle cell (VSMC), LDL exhibited transient CREB phosphorylation, & transiently increased activation of Akt and P38-, ERK-and JNK-MAPK pathways at 15 min. These transient CREB activation was returned to normal at 6 - 48 h but this phenomenon did not change the CREB expression [138]. Our data showed that CREB phosphorylation was decreased at 1 h after native LDL treatment. It is possible that LDL was metabolized by lipoprotein lipase to release fatty acid that can induce CREB activation. Free fatty acid have demonstrated acute activation of CREB via a protein kinase C (PKC) pathway in vascular smooth muscle cell (VSMC) [139]. Lipoprotein lipase enzyme plays a major role in the metabolism and transport of lipids in peripheral tissues and also presented in neuronal cells [140]. However, LDLR receptor knockout mice fed with high fat/cholesterol diet showed loss of nuclear CREB [138]. These data suggest that elevated cholesterol contribute to the decrease nuclear CREB expression [138]. It is possible that lipid in LDL particles might differently mediate CREB activation.

From our data, fox-LDL rapidly decrease pCREB and total CREB at 15 min whereas mox-LDL transiently activated nuclear translocations and activation of CREB. Different degree of LDL oxidation or oxidative products in oxidized LDL differently affect CREB phosphorylation in neuron. $H_2O_2$-induced ROS productions and pCREB were decreased within 1 h treatment, whereas the loss of total CREB protein was observed at 2 h in cerebellar granule neuron cells [69]. It is possibly due to the complex nature of CREB mediated gene expression and several factor such as phosphorylation at serine 133 [141]. CREB have been shown to promote cell survival, cell growth and mediate neuroprotective actions of growth factors [142, 143]. Thus, CREB play a multiple role in cellular response. It was reported that CREB induced the expression of bcl-2, an antiapoptotic gene to protect cells [138, 144]. This study demonstrated that high concentration of mox-LDL (100 μg/ml) increased neuronal apoptosis while subtoxic concentration of mox-LDL activated the CREB phosphorylation in neuroblastoma cells. This may be possible a mechanism for antiapoptotic effects in neuron. Oxidative stress have been reported to activate CREB

phosphorylation under certain experimental conditions. Acrolein and 4-HNE, an unsaturated aldehyde, stimulated the CREB phosphorylation and activated p38 MAPK [145]. In microglia cells, Aβ increased CREB phosphorylation through activation of Rsk2, a kinase downstream of Erk [146]. Our data indicated that lipid/cholesterol and LDL containing low level of oxidized LDL are able to induce CREB activation, whereas LDL containing high oxidized LDL might seem decrease CREB phosphorylation and expression due to the ROS productions.

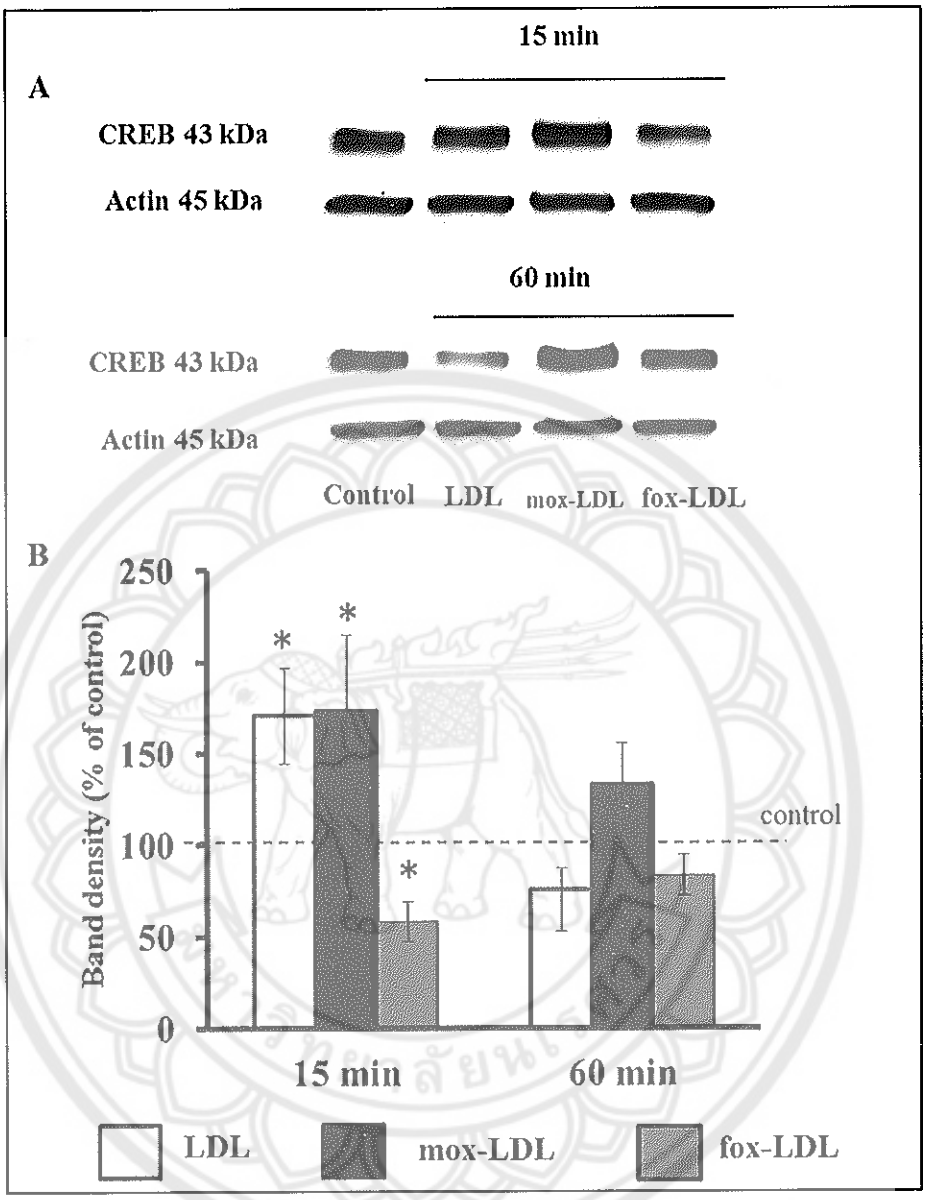



**Figure 33 Effect of time of LDL and oxidized LDL on nuclear CREB level**

**Note:** Cells were treated with 10 μg/ml of LDL, mox- and, fox-LDL for 15 and 1 h and nuclear protein was prepared by nuclear extraction kit. The protein concentrations were determined by BCA assay kit. The protein (20 μg/ml) was loaded and detected using anti-CREB (1:1000). The band density was visualized with ChemidocTMXRS and analyzed with Image LabTM software. Value present as mean ± SD of three independent experiments. (* $p \leq 0.05$ compared with control)

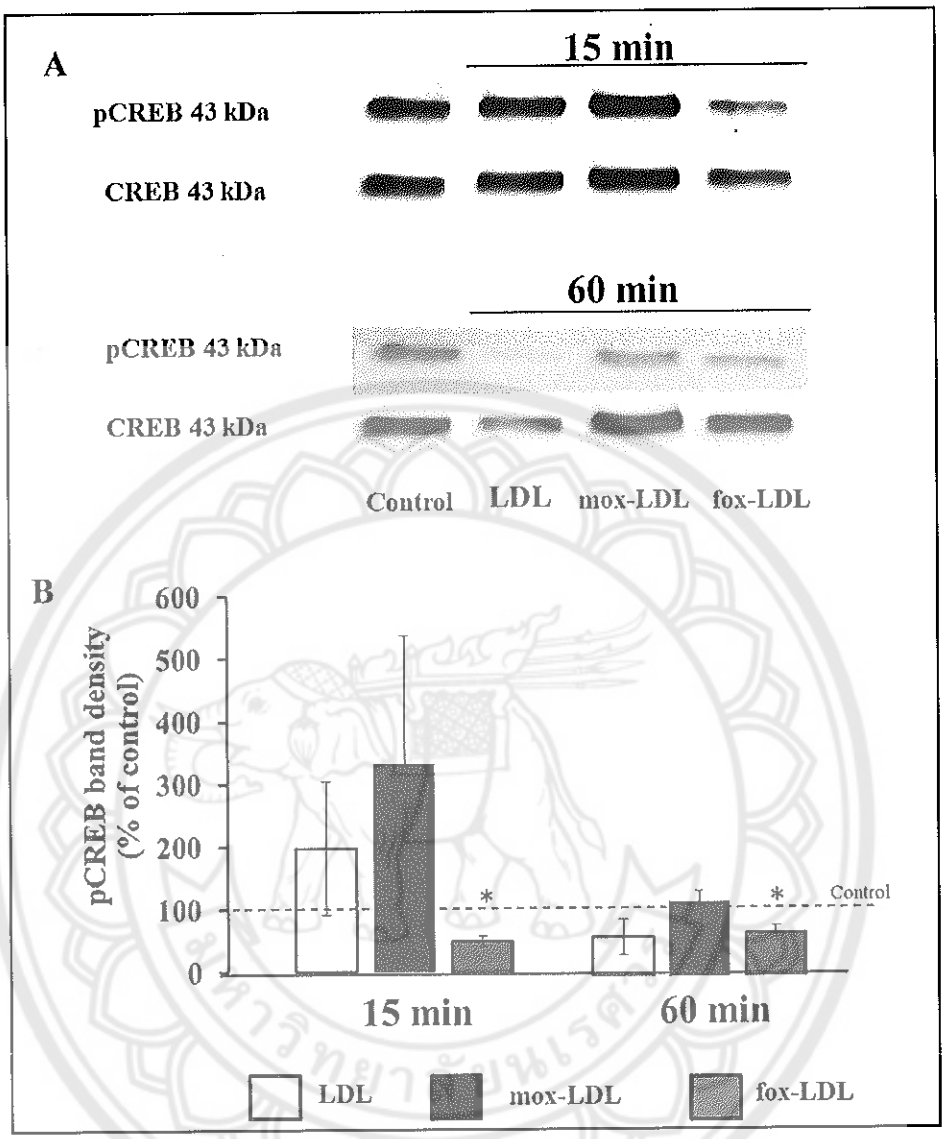



**Figure 34 Effect of time of LDL and its oxidation on phosphorylation of CREB**

**Note:** Cells were treated with 10 μg/ml of LDL, mox- and, fox-LDL for 15 and 1 h min and nuclear protein was prepared by nuclear extraction kit. The protein concentrations were determined by BCA assay kit. The nuclear protein (20 μg/ml) was load and blotted using anti-pCREB (1:1000). The band density was visualized with ChemidocTMXRS and analyzed with Image LabTM software. Value present as mean ± SD of three independent experiments. (* $p \leq 0.05$ compared with control)

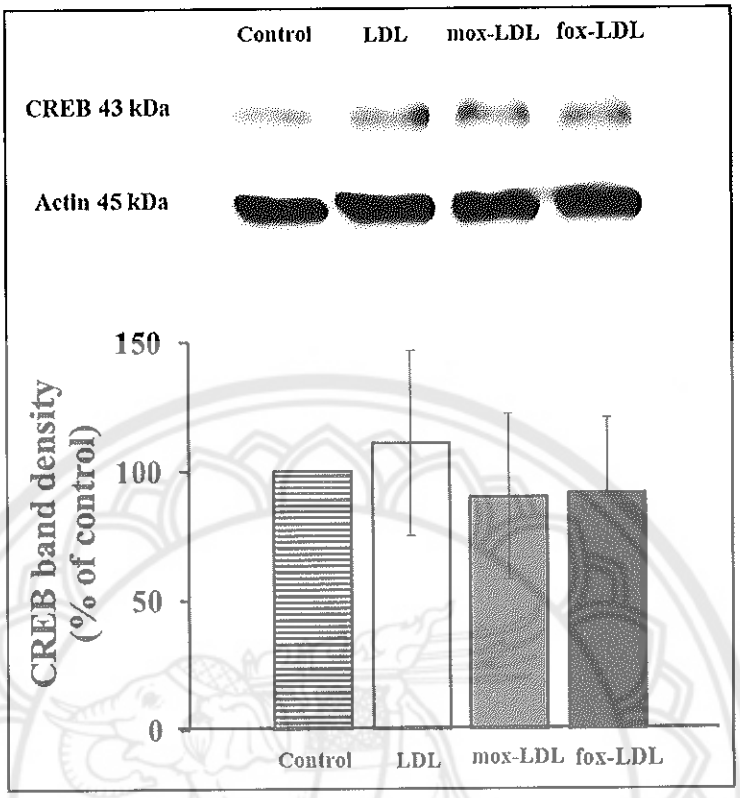



**Figure 35 Effect of LDL and oxidized LDL on CREB expression**

**Note:** SH-SY5Y cells were treated with 10 μg/ml of LDL, mox- and, fox-LDL for 24 h and cells were lysed by lysis buffer. The protein concentrations were determined by BCA assay kit. The protein (40 μg/ml) was loaded and detected using anti-CREB (1:1000). The band density was visualized with ChemidocTMXRS and analyzed with Image LabTM software. Value present as mean ± SD of three independent experiments

# CHAPTER V

# CONCLUSION

Development of AD is involved with multifaceted processes and thus only hypercholesterolemia might not be sufficient to induce the neurodegenerative disease but in concomitant with oxidative stress, it is possibly increases risk of AD. This study demonstrates that oxidized LDL induced cell death which was depending on the elevated levels of intracellular ROS. Oxidized LDL but not native LDL apparently increase the activity of AChE in SH-SY5Y neuroblastoma cells and this phenomenon was well correlation with intracellular ROS. The present study also demonstrated that the degree of LDL oxidation determined direction of cell death pathway. Necrotic cell damage was mainly mediated by LDL containing high concentration of oxidized lipid effecting LDH release. On the other hand, LDL contained low level of oxidized lipid led to apoptotic death by increasing caspase-3 activity. Interestingly, native LDL promoted APP processing to amyloidogenic pathway without affecting total APP expression in SH-SY5Y cells. Oxidized LDL herein failed to induce the secretion of APP production but enhanced the AChE activity. In addition, all three sample LDL preparations was not changed BACE-1 activity. Native LDL and mox-LDL rapidly increased CREB phosphorylation and this effect was decrease at 1 h. On the other hand, fox-LDL constantly decreased CREB phosphorylation due to the ROS productions and lead to the loss of neuronal plasticity.

Our study suggest that LDL and oxidized LDL mediated AD pathogenesis via different pathways. Elevated serum LDL possibly involves in the development of AD. Under the circumstance of oxidative stress, the formation of oxidized LDL may aggravate the progression of AD pathological processes in hypercholesterolemia. This finding support the roles of hypercholesterolemia and oxidative stress in pathological process of AD. Keeping low LDL level in the circulation and balancing between anti-oxidant and oxidation may be potential strategies to prevent or delay AD neurodegeneration.

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# APPENDIX

**Table 5 Effect of LDL on cell viability after 1 h treatment**

| Concentrations (μg/ml) | % cell viability | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|
| | No.1 | No.2 | No.3 | | | |
| PBS | 97.96 | 110.67 | 102.84 | 103.82 | 6.41 | 4.53 |
| NEO | 103.63 | 105.13 | 99.66 | 102.81 | 2.82 | 2.00 |
| Control | 100.01 | 100.01 | 100.00 | 100.00 | 0.00 | 0.00 |
| 10 | 100.04 | 87.48 | 97.99 | 95.17 | 6.74 | 4.77 |
| 25 | 96.45 | 88.93 | 101.81 | 95.73 | 6.47 | 4.57 |
| 50 | 95.37 | 88.93 | 100.29 | 94.86 | 5.70 | 4.03 |
| 100 | 92.69 | 86.87 | 92.62 | 90.72 | 3.34 | 2.36 |
| 200 | 91.28 | - | 103.00 | 83.76 | 8.29 | 5.86 |

**Table 6 Effect of mox-LDL on Cell viability after 1 h treatment**

| Concentrations (μg/ml) | % cell viability | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|
| | No.1 | No.2 | No.3 | | | |
| PBS | 97.96 | 110.67 | 102.84 | 103.82 | 6.41 | 4.53 |
| NEO | 103.63 | 105.13 | 99.66 | 102.81 | 2.82 | 2.00 |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 | 0.00 |
| 10 | 96.25 | 97.79 | 94.67 | 96.24 | 1.56 | 1.10 |
| 25 | 99.34 | 92.21 | 114.97 | 102.17 | 11.64 | 8.23 |
| 50 | 95.71 | 93.13 | 111.17 | 100.00 | 9.75 | 6.90 |
| 100 | 91.54 | 89.24 | 109.65 | 96.81 | 11.18 | 7.90 |
| 200 | 89.46 | 85.11 | 98.20 | 90.92 | 6.67 | 4.71 |

**Table 7 Effect of fox-LDL on Cell viability after1 h treatment**

| Concentrations | % cell viability | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | | | |
| PBS | 97.96 | 110.67 | 102.84 | 103.82 | 6.41 | 4.53 |
| NEO | 103.63 | 105.13 | 99.66 | 102.81 | 2.82 | 2.00 |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 | 0.00 |
| 10 | 95.10 | 96.42 | 126.76 | 106.09 | 17.91 | 12.66 |
| 25 | 93.36 | 93.97 | 96.43 | 94.59 | 1.63 | 1.15 |
| 50 | 88.05 | 87.55 | - | 87.80 | 0.35 | 0.25 |
| 100 | 84.63 | 76.93 | 97.67 | 86.41 | 10.48 | 7.41 |
| 200 | 80.06 | 53.94 | 82.97 | 72.32 | 15.99 | 11.31 |

**Table 8 Effect of nLDL on Cell viability after 2 h treatment**

| Concentrations | % cell viability | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | | | |
| PBS | 98.87 | 102.73 | 97.09 | 99.56 | 2.88 | 2.04 |
| NEO | 105.58 | 101.64 | 88.11 | 98.44 | 9.16 | 6.48 |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 | 0.00 |
| 10 | 103.53 | 103.52 | 97.53 | 101.53 | 3.46 | 2.45 |
| 25 | 100.01 | 105.55 | 92.47 | 99.35 | 6.56 | 4.64 |
| 50 | 94.62 | 103.20 | 97.96 | 98.59 | 4.32 | 3.06 |
| 100 | 81.41 | 95.29 | 98.34 | 91.68 | 9.02 | 6.38 |
| 200 | 72.61 | 90.52 | 95.22 | 86.11 | 11.93 | 8.44 |

**Table 9 Effect of mox-LDL on Cell viability after 2 h treatment**

| Concentrations (µg/ml) | % cell viability | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|
| | No.1 | No.2 | No.3 | | | |
| PBS | 98.87 | 102.73 | 97.09 | 99.56 | 2.88 | 2.04 |
| NEO | 105.58 | 101.64 | 88.11 | 98.44 | 9.16 | 6.48 |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 | 0.00 |
| 10 | 91.45 | 101.25 | 92.55 | 95.08 | 5.37 | 3.79 |
| 25 | 96.16 | 100.15 | 98.38 | 98.23 | 2.00 | 1.41 |
| 50 | 108.55 | 96.78 | 92.32 | 99.22 | 8.39 | 5.93 |
| 100 | 98.21 | 96.63 | 100.12 | 98.32 | 1.75 | 1.24 |
| 200 | 93.09 | 90.05 | 96.72 | 93.29 | 3.34 | 2.36 |

**Table 10 Effect of fox-LDL on Cell viability after 2 h treatment**

| Concentrations (µg/ml) | % cell viability | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|
| | No.1 | No.2 | No.3 | | | |
| PBS | 98.87 | 102.73 | 97.09 | 99.56 | 2.88 | 2.04 |
| NEO | 105.58 | 101.64 | 88.11 | 98.44 | 9.16 | 6.48 |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 | 0.00 |
| 10 | 110.70 | 105.24 | 106.88 | 107.61 | 2.80 | 1.98 |
| 25 | 90.22 | 101.25 | 100.51 | 97.32 | 6.16 | 4.36 |
| 50 | 92.06 | 94.35 | 100.93 | 95.78 | 4.60 | 3.25 |
| 100 | 80.59 | 74.54 | 100.58 | 85.24 | 13.63 | 9.64 |
| 200 | 60.73 | 61.70 | 84.29 | 68.91 | 13.33 | 9.43 |

**Table 11 Effect of nLDL on Cell viability after 4 h treatment**

| Concentrations (µg/ml) | % cell viability | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|
| | No.1 | No.2 | No.3 | | | |
| **PBS** | 101.80 | 102.46 | 107.23 | 103.83 | 2.96 | 2.09 |
| **NEO** | 113.98 | 94.60 | 101.14 | 103.24 | 9.86 | 6.97 |
| **Control** | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 | 0.00 |
| **10** | 94.08 | 102.84 | 101.11 | 99.34 | 4.64 | 3.28 |
| **25** | 102.70 | 99.43 | 102.88 | 101.67 | 1.94 | 1.37 |
| **50** | 97.81 | 102.65 | 103.00 | 101.15 | 2.90 | 2.05 |
| **100** | 80.78 | 102.46 | 100.19 | 94.48 | 11.92 | 8.43 |
| **200** | 82.80 | 90.34 | - | 86.57 | 5.33 | 3.77 |

**Table 12 Effect of mox-LDL on Cell viability after 4 h treatment**

| Concentrations (µg/ml) | % cell viability | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|
| | No.1 | No.2 | No.3 | | | |
| **PBS** | 101.80 | 102.46 | 107.23 | 103.83 | 2.96 | 2.09 |
| **NEO** | 113.98 | 94.60 | 101.14 | 103.24 | 9.86 | 6.97 |
| **Control** | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 | 0.00 |
| **10** | 104.94 | 93.84 | 106.05 | 101.61 | 6.75 | 4.77 |
| **25** | 91.00 | 105.11 | 108.57 | 101.56 | 9.31 | 6.58 |
| **50** | 114.84 | 93.28 | 105.04 | 104.39 | 10.79 | 7.63 |
| **100** | 85.89 | 91.95 | 98.55 | 92.13 | 6.34 | 4.48 |
| **200** | 76.42 | 83.52 | 107.53 | 89.15 | 16.30 | 11.53 |

**Table 13 Effect of fox-LDL on Cell viability after 4 h treatment**

| Concentrations | % cell viability | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | | | |
| PBS | 101.80 | 102.46 | 107.23 | 103.83 | 2.96 | 2.09 |
| NEO | 113.98 | 94.60 | 101.14 | 103.24 | 9.86 | 6.97 |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 | 0.00 |
| 10 | 107.17 | 92.23 | 109.10 | 102.84 | 9.23 | 6.53 |
| 25 | 88.34 | 92.42 | 105.55 | 95.44 | 9.00 | 6.36 |
| 50 | 101.21 | 73.58 | 109.03 | 94.61 | 18.63 | 13.17 |
| 100 | 79.50 | 62.22 | 92.73 | 78.15 | 15.30 | 10.82 |
| 200 | 70.88 | 44.32 | - | 57.60 | 18.78 | 13.28 |

**Table 14 Effect of nLDL on Cell viability after 24 h treatment**

| Concentrations | % cell viability | | | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | | | |
| PBS | 94.25 | 85.59 | 82.67 | 96.80 | 102.49 | 94.25 | 92.67 | 7.33 | 3.28 |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0 | 0 |
| 10 | 95.97 | - | 105.65 | - | 100.98 | 95.97 | 99.64 | 4.65 | 2.68 |
| 25 | 89.87 | 75.95 | 111.90 | 122.14 | 101.03 | 89.87 | 98.46 | 16.75 | 7.49 |
| 50 | 90.58 | 86.72 | 78.12 | 97.55 | 114.13 | 90.58 | 92.95 | 12.16 | 5.44 |
| 100 | 86.16 | 81.20 | 80.40 | 92.66 | 101.03 | 86.16 | 87.94 | 7.77 | 3.48 |
| 200 | 84.22 | 89.23 | 71.28 | 69.62 | 75.60 | 84.22 | 79.03 | 7.98 | 3.57 |

**Table 15 Effect of mox-LDL on Cell viability after 24 h treatment**

| Concentrations | % cell viability | | | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | | | |
| PBS | 94.25 | 85.59 | 82.67 | 96.80 | 102.49 | 94.25 | 92.67 | 7.33 | 3.28 |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0 | 0 |
| 10 | 86.60 | - | 108.71 | 114.40 | 103.72 | 86.60 | 100.01 | 12.81 | 5.73 |
| 25 | 82.54 | 93.65 | 91.62 | 101.21 | 115.26 | 82.54 | 94.47 | 12.42 | 5.55 |
| 50 | 72.64 | 76.97 | 77.32 | 88.29 | 111.52 | 72.64 | 83.23 | 15.00 | 6.71 |
| 100 | 58.59 | 88.24 | 69.33 | 90.32 | 87.10 | 58.59 | 75.36 | 15.01 | 6.71 |
| 200 | 32.87 | 95.18 | 55.21 | 55.15 | 89.88 | 32.87 | 60.19 | 27.01 | 12.08 |

**Table 16 Effect of fox-LDL on Cell viability after 24 h treatment**

| Concentrations | % cell viability | | | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | | | |
| PBS | 94.25 | 85.59 | 82.67 | 96.80 | 102.49 | 94.25 | 92.67 | 7.33 | 3.28 |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0 | 0 |
| 10 | 82.27 | 73.71 | 89.40 | 130.99 | 105.92 | 82.27 | 94.09 | 21.06 | 9.42 |
| 25 | 87.40 | 74.02 | 109.81 | 99.68 | 118.58 | 87.40 | 96.15 | 16.40 | 7.34 |
| 50 | 83.42 | 83.42 | 76.19 | 87.44 | 77.03 | 83.42 | 81.82 | 4.33 | 1.94 |
| 100 | 24.04 | 76.69 | 46.84 | 61.83 | 10.49 | 24.04 | 40.65 | 25.48 | 11.40 |
| 200 | 13.61 | 43.44 | 19.30 | 14.76 | 1.69 | 13.61 | 17.73 | 13.88 | 6.21 |

**Table 17 Effect of nLDL on intracellular ROS at 1 h treatment**

| Concentrations | % Intracellular ROS | | | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0 | 0 |
| 10 | 96.87 | 106.98 | 99.82 | 107.18 | 110.33 | 107.18 | 104.73 | 5.18 | 1.83 |
| 25 | 103.94 | 100.28 | 98.19 | 110.73 | 110.73 | 99.24 | 103.85 | 5.67 | 2.00 |
| 50 | 100.99 | 94.56 | 93.82 | 103.31 | 106.47 | 107.52 | 101.11 | 5.84 | 2.07 |
| 100 | 102.36 | 99.32 | 91.49 | 106.92 | 110.23 | 103.61 | 102.32 | 6.51 | 2.30 |
| 200 | 98.79 | 91.13 | 71.03 | 87.50 | 85.45 | 137.95 | 95.31 | 22.78 | 8.05 |

**Table 18 Effect of mox-LDL on intracellular ROS at 1 h treatment**

| Concentrations | % Intracellular ROS | | | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0 | 0 |
| 10 | 102.72 | 99.78 | 110.81 | 107.83 | 124.69 | 109.10 | 108.53 | 8.08 | 2.86 |
| 25 | 105.24 | 93.68 | 108.85 | 108.14 | 146.81 | 114.51 | 111.34 | 16.92 | 5.98 |
| 50 | 104.67 | 97.01 | 114.56 | 101.30 | 160.00 | 134.00 | 115.81 | 23.28 | 8.23 |
| 100 | 109.73 | 106.38 | 123.97 | 112.72 | 183.87 | 148.75 | 128.05 | 28.56 | 10.10 |
| 200 | 145.77 | 130.03 | 154.32 | 128.49 | 179.27 | 162.77 | 147.58 | 19.09 | 6.75 |

**Table 19 Effect of fox-LDL on intracellular ROS at 1 h treatment**

| Concentrations | % Intracellular ROS | | | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0 | 0 |
| 10 | 101.10 | 90.88 | 107.45 | 121.59 | 115.94 | 120.65 | 109.60 | 12.11 | 4.28 |
| 25 | 109.33 | 100.49 | 134.27 | 134.27 | 167.04 | 152.24 | 132.94 | 25.10 | 8.87 |
| 50 | 112.23 | 105.06 | 136.42 | 141.62 | 186.40 | 158.29 | 140.00 | 29.98 | 10.60 |
| 100 | 132.56 | 122.21 | 148.13 | 165.49 | 219.89 | 194.42 | 163.78 | 37.52 | 13.26 |
| 200 | 168.29 | 146.82 | 171.45 | 186.66 | 261.32 | 163.15 | 182.95 | 40.50 | 14.32 |

**Table 20 Effect of nLDL on intracellular ROS at 2 h treatment**

| Concentrations | % Intracellular ROS | | | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0 | 0 |
| 10 | 92.95 | 100.75 | 94.56 | 99.48 | 101.45 | 95.54 | 97.45 | 3.56 | 1.26 |
| 25 | 95.09 | 96.24 | 95.63 | 108.71 | 101.66 | 89.59 | 97.82 | 6.57 | 2.32 |
| 50 | 92.65 | 92.96 | 90.27 | 98.38 | 100.41 | 94.33 | 94.83 | 3.82 | 1.35 |
| 100 | 103.58 | 96.02 | 87.26 | 103.62 | 100.35 | 89.44 | 96.71 | 7.08 | 2.50 |
| 200 | 109.96 | 101.19 | 76.64 | 95.22 | 89.42 | 77.80 | 91.71 | 13.12 | 4.64 |

**Table 21 Effect of mox-LDL on intracellular ROS at 2 h treatment**

| Concentrations | % Intracellular ROS | | | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0 | 0 |
| 10 | 104.29 | 104.72 | 114.57 | 113.37 | 138.83 | 119.90 | 114.94 | 11.91 | 4.86 |
| 25 | 117.79 | 108.73 | 120.08 | 114.51 | 186.30 | 139.47 | 128.93 | 27.07 | 11.05 |
| 50 | 114.73 | 112.20 | 136.32 | 110.20 | 214.68 | 167.31 | 138.27 | 39.52 | 16.14 |
| 100 | 122.41 | 133.98 | 140.20 | 125.32 | - | 189.18 | 139.60 | 25.14 | 10.26 |
| 200 | 176.47 | 166.76 | 188.55 | 149.57 | 227.26 | 197.24 | 180.58 | 26.43 | 10.79 |

**Table 22 Effect of fox-LDL on intracellular ROS at 2 h treatment**

| Concentrations | % Intracellular ROS | | | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0 | 0 |
| 10 | 104.38 | 90.67 | 116.17 | 122.67 | 119.88 | 123.60 | 112.89 | 12.93 | 4.57 |
| 25 | 107.60 | 102.54 | 146.93 | 146.93 | 176.73 | 162.34 | 140.51 | 29.65 | 10.48 |
| 50 | 114.98 | 111.43 | 157.65 | 160.78 | 207.76 | 183.75 | 156.06 | 37.80 | 13.36 |
| 100 | 142.82 | 132.42 | 166.59 | 183.01 | 244.02 | 228.77 | 182.94 | 45.30 | 16.02 |
| 200 | 189.24 | 161.59 | 193.02 | 206.08 | 304.76 | 204.63 | 209.88 | 49.17 | 17.38 |

**Table 23 Effect of nLDL on intracellular ROS at 4 h treatment**

| Concentrations | % Intracellular ROS | | | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0 | 0 |
| 10 | 95.46 | 104.69 | 98.65 | 100.66 | 102.67 | 97.64 | 99.96 | 3.39 | 1.20 |
| 25 | 94.30 | 93.73 | 95.41 | 105.24 | 97.37 | 88.52 | 95.76 | 5.50 | 1.94 |
| 50 | 91.29 | 87.69 | 89.96 | 94.91 | 95.90 | 90.95 | 91.78 | 3.09 | 1.09 |
| 100 | 97.90 | 92.45 | 86.79 | 98.44 | 94.20 | 84.68 | 92.41 | 5.67 | 2.01 |
| 200 | 111.47 | 98.63 | 77.39 | 93.56 | 85.48 | 75.08 | 90.27 | 13.78 | 4.87 |

**Table 24 Effect of mox-LDL on intracellular ROS at 4 h treatment**

| Concentrations | % Intracellular ROS | | | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0 | 0 |
| 10 | 101.33 | 99.06 | 109.31 | 116.61 | 112.02 | 101.33 | 99.06 | 109.31 | 116.61 |
| 25 | 134.40 | 110.83 | 120.99 | 134.79 | 135.56 | 134.40 | 110.83 | 120.99 | 134.79 |
| 50 | 134.59 | 118.49 | 136.88 | 135.87 | 136.13 | 134.59 | 118.49 | 136.88 | 135.87 |
| 100 | 162.10 | 157.28 | 160.18 | 178.47 | 183.66 | 162.10 | 157.28 | 160.18 | 178.47 |
| 200 | 259.14 | 193.90 | 227.54 | 215.18 | 247.93 | 259.14 | 193.90 | 227.54 | 215.18 |

**Table 25 Effect of fox-LDL on intracellular ROS at 4 h treatment**

| Concentrations | % Intracellular ROS | | | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0 | 0 |
| 10 | 95.93 | 94.49 | 108.69 | 128.36 | 129.33 | 131.28 | 114.68 | 17.16 | 6.49 |
| 25 | 115.68 | 103.26 | 116.91 | 159.27 | 158.22 | 168.70 | 137.01 | 28.10 | 10.62 |
| 50 | 116.13 | 112.43 | 129.47 | 173.35 | 178.63 | 192.37 | 150.40 | 35.04 | 13.24 |
| 100 | 154.36 | 157.83 | 184.17 | 197.06 | 208.58 | 261.04 | 193.84 | 39.22 | 14.82 |
| 200 | 232.45 | 222.14 | 261.54 | 250.00 | 262.15 | 251.74 | 246.67 | 16.13 | 6.10 |

**Table 26 Effect of nLDL on intracellular ROS at 24 h treatment**

| Concentrations | % Intracellular ROS | | | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0 | 0 |
| 10 | 124.90 | 146.52 | 98.35 | 100.36 | 103.37 | 95.34 | 111.47 | 20.15 | 7.12 |
| 25 | 139.97 | 123.89 | 108.67 | 101.56 | 98.52 | 94.45 | 111.18 | 17.49 | 6.18 |
| 50 | 160.85 | 132.86 | 104.36 | 102.21 | 102.21 | 100.06 | 117.09 | 24.73 | 8.74 |
| 100 | 173.08 | 154.09 | 101.21 | 93.25 | 95.52 | 85.29 | 117.07 | 36.88 | 13.04 |
| 200 | 197.40 | 170.29 | 92.37 | 79.72 | 82.25 | 77.19 | 116.54 | 53.09 | 18.77 |

**Table 27 Effect of mox-LDL on intracellular ROS at 24 h treatment**

| Concentrations | % Intracellular ROS | | | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | - | 100.00 | 0 | 0 |
| 10 | 138.92 | 129.95 | 163.35 | 143.65 | 132.57 | - | 141.69 | 13.24 | 7.65 |
| 25 | 204.39 | 159.13 | 197.84 | 177.68 | 171.10 | - | 182.03 | 18.79 | 10.85 |
| 50 | 199.02 | 179.07 | 232.88 | 183.45 | 176.07 | - | 194.10 | 23.41 | 13.52 |
| 100 | 211.37 | 209.09 | 216.94 | 206.47 | 201.89 | - | 209.15 | 5.60 | 3.23 |
| 200 | 360.91 | 278.75 | 343.17 | 275.69 | 292.23 | - | 310.15 | 39.25 | 22.66 |

**Table 28 Effect of fox-LDL on intracellular ROS at 24 h treatment**

| Concentrations | % Intracellular ROS | | | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | | | |
| Control | 100.00 | - | 100.00 | 100.00 | 100.00 | - | 100.00 | 0 | 0 |
| 10 | 129.26 | - | 131.78 | 132.85 | 122.22 | 130.72 | 129.36 | 4.21 | 2.10 |
| 25 | 124.32 | - | 147.69 | 154.97 | 152.89 | 149.77 | 145.93 | 12.40 | 6.20 |
| 50 | 167.16 | - | 207.77 | 211.44 | 219.99 | 202.88 | 201.85 | 20.38 | 10.19 |
| 100 | 375.40 | - | 437.84 | 423.08 | 509.18 | 494.42 | 447.98 | 54.53 | 27.26 |
| 200 | 933.53 | - | 1031.88 | 998.04 | 1122.10 | 924.74 | 1002.06 | 80.60 | 40.30 |

**Table 29 Effect of nLDL on cellular AChE activtity after 1 h treatment**

| Concentrations | % Cellular AChE activity | | | | | | Mean | SEM |
|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | | |
| control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | - | 100.00 | 0.00 |
| Neo (100µg/ml) | 1.54 | 1.39 | 7.89 | 5.00 | 3.95 | - | 3.81 | 1.09 |
| 10 | 96.89 | - | 138.26 | 120.23 | 139.70 | - | 114.67 | 7.83 |
| 25 | 81.45 | 111.28 | 105.38 | 121.03 | 122.53 | - | 99.91 | 7.04 |
| 50 | 97.49 | 87.69 | 113.73 | 105.41 | 111.62 | - | 97.50 | 4.31 |
| 100 | 97.57 | 102.87 | 140.20 | 113.93 | 132.15 | - | 107.58 | 7.38 |
| 200 | 99.00 | 60.19 | 97.53 | 108.39 | 96.76 | - | 87.34 | 7.99 |

**Table 30 Effect of mox-LDL on cellular AChE activtity after 1 h treatment**

| Concentrations | AChE activity (% of control) | | | | | | Mean | SEM |
|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| Neo (100 µg/ml) | 1.54 | 1.39 | 7.89 | 5.00 | 3.95 | 3.10 | 3.81 | 1.09 |
| 10 | 139.85 | 161.77 | 137.03 | - | 130.34 | 122.39 | 138.27 | 6.60 |
| 25 | 127.58 | 153.36 | 138.80 | 151.98 | 94.43 | 115.28 | 130.23 | 10.18 |
| 50 | 128.20 | 197.91 | 144.18 | 110.58 | 119.29 | 113.45 | 135.60 | 14.69 |
| 100 | 156.16 | 93.67 | 170.41 | 168.92 | 138.63 | 112.73 | 140.09 | 14.02 |
| 200 | 94.91 | 119.76 | 178.26 | 195.38 | 107.09 | 113.68 | 134.84 | 18.54 |

**Table 31 Effect of fox-LDL on cellular AChE activtity after 1 h treatment**

| Concentration | % cellular AChE activity | | | | | | Mean | SEM |
|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| Neo (100 µg/ml) | 1.54 | 1.39 | 7.89 | 5.00 | 3.95 | 3.10 | 3.81 | 1.09 |
| 10 | 116.47 | 213.90 | 112.26 | 134.17 | 106.66 | - | 136.69 | 19.84 |
| 25 | 101.46 | 149.92 | 178.62 | 156.72 | 111.28 | 125.03 | 137.17 | 13.20 |
| 50 | 107.98 | 133.52 | 169.40 | 121.65 | 111.64 | 117.46 | 126.94 | 10.11 |
| 100 | 125.05 | 108.00 | 168.90 | 202.86 | 124.86 | 126.30 | 142.66 | 16.01 |
| 200 | 179.59 | 113.48 | 190.69 | 136.63 | 149.17 | 130.95 | 150.09 | 13.28 |

**Table 32 Effect of nLDL on cellular AChE activtity after 2 h treatment**

| Concentrations | % cellular AChE activity | | | | | | | Mean | SEM |
|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | No.7 | | |
| control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100 | 0.00 |
| Neo (100 µg/ml) | 2.86 | 3.04 | 4.56 | 9.65 | 1.91 | 0.72 | - | 2.86 | 3.04 |
| 10 | 56.28 | 83.57 | 80.58 | 111.11 | 134.88 | 76.52 | 92.82 | 90.82 | 10.42 |
| 25 | 54.64 | 149.46 | 86.93 | 80.84 | 142.33 | 110.07 | 102.18 | 103.78 | 13.78 |
| 50 | 57.95 | 107.57 | 119.86 | 69.86 | 133.08 | 125.50 | 103.26 | 102.44 | 11.60 |
| 100 | 40.51 | 128.90 | 99.15 | 63.14 | 134.08 | 139.67 | 102.79 | 101.18 | 15.34 |
| 200 | 43.13 | 116.12 | 100.29 | 63.51 | 130.91 | 142.86 | 105.21 | 100.29 | 14.57 |

**Table 33 Effect of mox-LDL on cellular AChE activtity after 2 h treatment**

| Concentrations | % cellular AChE activity | | | | | | | Mean | SEM |
|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | No.7 | | |
| control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| Neo (100µg/ml) | 2.86 | 3.04 | 4.56 | 9.65 | 1.91 | 0.72 | 1.26 | 3.43 | 1.23 |
| 10 | - | 140.22 | 138.63 | 133.09 | 117.90 | 107.44 | 117.57 | 125.81 | 5.46 |
| 25 | 151.24 | 123.39 | 111.05 | 124.63 | 132.27 | 149.40 | 111.88 | 129.12 | 6.64 |
| 50 | 167.02 | 125.22 | 123.69 | 114.85 | 118.12 | 149.37 | 109.34 | 129.66 | 8.51 |
| 100 | 151.11 | 203.42 | 92.46 | 165.25 | 118.55 | 152.20 | 104.58 | 141.08 | 15.72 |
| 200 | 162.32 | 178.66 | 151.05 | 114.55 | 127.68 | 159.63 | 95.56 | 141.35 | 12.09 |

**Table 34 Effect of fox-LDL on cellular AChE activtity after 2 h treatment**

| Concentrations | % cellular AChE activity | | | | | | | Mean | SEM |
|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | No.7 | | |
| control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| Neo (100µg/ml) | 2.86 | 3.04 | 4.56 | 9.65 | 1.91 | 0.72 | 1.26 | 3.43 | 1.23 |
| 10 | 164.62 | 126.72 | 130.94 | 94.42 | 117.79 | 74.88 | 164.62 | 114.22 | 12.37 |
| 25 | 170.27 | 127.65 | 130.77 | 99.22 | 128.27 | 115.31 | 170.27 | 125.57 | 9.37 |
| 50 | 190.90 | 136.04 | 137.62 | 152.18 | 136.98 | 131.44 | 190.90 | 140.62 | 11.19 |
| 100 | 207.81 | 124.42 | 133.31 | 158.65 | 145.71 | 150.23 | 207.81 | 150.84 | 11.27 |
| 200 | 161.71 | 193.50 | 151.72 | 166.71 | 133.15 | 171.22 | 161.71 | 163.91 | 7.58 |

**Table 35 Effect of nLDL on cellular AChE activtity after 4 h treatment**

| Concentrations | % cellular AChE activity | | | | | | | | Mean | SEM |
|---|---|---|---|---|---|---|---|---|---|---|
| (µg/ml) | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | No.7 | No.8 | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| Neo (100µg/ml) | 6.47 | 1.85 | 8.49 | 6.43 | 1.62 | 3.53 | 13.35 | 3.77 | 5.69 | 1.38 |
| 10 | 73.07 | 94.85 | 60.61 | 98.14 | 145.39 | 94.04 | 100.20 | 110.89 | 97.15 | 8.92 |
| 25 | 133.13 | 91.33 | 11.61 | 94.20 | 155.77 | 102.17 | 94.83 | 119.67 | 100.34 | 14.97 |
| 50 | 75.24 | 78.88 | 78.01 | 106.84 | 106.25 | 106.52 | 102.01 | 113.82 | 95.95 | 5.56 |
| 100 | 116.10 | 58.571 | 117.45 | 67.53 | 212.94 | 114.55 | 110.96 | 125.22 | 115.42 | 16.45 |
| 200 | 86.153 | 45.16 | 116.12 | 71.49 | 160.37 | 121.37 | 108.74 | 135.05 | 105.56 | 12.98 |

**Table 36 Effect of mox-LDL on cellular AChE activtity after 4 h treatment**

| Concentrations (μg/ml) | % cellular AChE activity | | | | | | | | Mean | SEM |
|---|---|---|---|---|---|---|---|---|---|---|
| | No.1 | N.o2 | No.3 | No.4 | No.5 | No.6 | No.7 | No.8 | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| Neo (100μg/ml) | 6.47 | 1.85 | 8.49 | 6.43 | 1.62 | 3.53 | 13.35 | 3.77 | 5.69 | 1.38 |
| 10 | 230.69 | 114.91 | 234.61 | 121.64 | 141.17 | 95.65 | 105.78 | 106.42 | 143.86 | 19.95 |
| 25 | 243.01 | 116.87 | 140.95 | 114.11 | 113.60 | 67.01 | 103.56 | 99.78 | 124.86 | 18.40 |
| 50 | 286.10 | 98.92 | 201.57 | 125.54 | 130.67 | 98.76 | 93.79 | 97.98 | 141.67 | 24.16 |
| 100 | 255.91 | 120.54 | 226.89 | 129.58 | 131.86 | 111.42 | 101.12 | 107.76 | 148.14 | 20.86 |
| 200 | 127.94 | 140.94 | 181.41 | 139.62 | 128.87 | 110.96 | 105.95 | 110.17 | 130.73 | 8.65 |

**Table 37 Effect of fox-LDL on cellular AChE activtity after 4 h treatment**

| Concentrations (μg/ml) | % cellular AChE activity | | | | | | | | Mean | SEM |
|---|---|---|---|---|---|---|---|---|---|---|
| | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | No.7 | No.8 | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| Neo (100μg/ml) | 6.47 | 1.85 | 8.49 | 6.43 | 1.62 | 3.53 | 13.35 | 3.77 | 5.69 | 1.38 |
| 10 | 161.87 | 98.099 | 150.12 | 119.72 | 118.72 | 238.96 | 83.59 | 88.63 | 129.67 | 17.19 |
| 25 | 159.09 | 152.22 | 197.59 | 152.11 | 115.76 | 183.48 | 108.42 | 95.62 | 144.53 | 12.02 |
| 50 | 202.07 | 116.00 | 152.58 | 136.35 | - | 325.63 | 111.78 | 115.26 | 163.39 | 25.41 |
| 100 | 143.80 | 119.48 | 234.63 | 120.23 | 119.53 | 402.22 | 124.97 | 119.34 | 168.43 | 33.64 |
| 200 | 262.16 | 163.93 | 290.25 | 177.66 | 262.47 | 400.82 | 160.439 | 145.96 | 221.31 | 31.53 |

**Table 38 Effect of nLDL on cellular AChE activtity after 24 h treatment**

| Concentrations (μg/ml) | % cellular AChE activity | | | | | | | | Mean | SEM |
|---|---|---|---|---|---|---|---|---|---|---|
| | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | No.7 | No.8 | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| Neo (100μg/ml) | 6.32 | 2.34 | 6.75 | 40.78 | 5.81 | 4.88 | 2.62 | 11.12 | 10.08 | 4.49 |
| 10 | 94.24 | 81.11 | 86.75 | 64.60 | - | 105.43 | 116.25 | 104.97 | 93.34 | 6.17 |
| 25 | 117.79 | 65.32 | 86.02 | 84.05 | 130.39 | 86.17 | 130.34 | 141.25 | 105.17 | 9.90 |
| 50 | 104.38 | 85.07 | 136.00 | 137.27 | 167.20 | 83.68 | 121.93 | 145.92 | 122.68 | 10.51 |
| 100 | 125.69 | 106.01 | 137.35 | 179.07 | 78.38 | 101.08 | 135.52 | 118.95 | 122.76 | 10.60 |
| 200 | 148.23 | 130.45 | 152.74 | 145.44 | 169.28 | 128.46 | 119.16 | 130.90 | 140.58 | 5.76 |

**Table 39 Effect of nLDL on cellular AChE activtity after 24 h treatment**

| Concentrations (μg/ml) | % cellular AChE activity | | | | | | | | Mean | SEM |
|---|---|---|---|---|---|---|---|---|---|---|
| | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | No.7 | No.8 | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| Neo (100μg/ml) | 6.32 | 2.34 | 6.75 | 40.78 | 5.81 | 4.88 | 2.62 | 11.12 | 10.08 | 4.49 |
| 10 | 123.08 | 148.64 | 106.99 | 118.38 | - | 87.37 | 130.07 | 136.77 | 121.62 | 7.12 |
| 25 | 117.77 | 89.91 | 109.90 | 183.72 | 130.17 | 109.60 | 143.72 | 131.41 | 127.02 | 9.98 |
| 50 | 181.66 | 99.15 | 147.37 | 211.29 | 170.48 | 138.96 | 157.27 | 136.72 | 155.36 | 11.87 |
| 100 | 196.29 | 139.04 | 185.64 | 233.36 | 188.28 | 142.08 | 172.61 | 155.57 | 176.61 | 11.09 |
| 200 | 202.33 | 228.24 | 236.73 | 307.44 | 240.20 | 145.15 | 131.01 | 194.77 | 210.73 | 19.90 |

**Table 40 Effect of mox-LDL on cellular AChE activtity after 24 h treatment**

| Concentrations (μg/ml) | % cellular AChE activity | | | | | | | | Mean | SEM |
|---|---|---|---|---|---|---|---|---|---|---|
| | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | No.7 | No.8 | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| Neo (100μg/ml) | 6.32 | 2.34 | 6.75 | 40.78 | 5.81 | 4.88 | 2.62 | 11.12 | 10.08 | 4.49 |
| 10 | 123.08 | 148.64 | 106.99 | 118.38 | - | 87.37 | 130.07 | 136.77 | 121.62 | 7.12 |
| 25 | 117.77 | 89.91 | 109.90 | 183.72 | 130.17 | 109.60 | 143.72 | 131.41 | 127.02 | 9.98 |
| 50 | 181.66 | 99.15 | 147.37 | 211.29 | 170.48 | 138.96 | 157.27 | 136.72 | 155.36 | 11.87 |
| 100 | 196.29 | 139.04 | 185.64 | 233.36 | 188.28 | 142.08 | 172.61 | 155.57 | 176.61 | 11.09 |
| 200 | 202.33 | 228.24 | 236.73 | 307.44 | 240.20 | 145.15 | 131.01 | 194.77 | 210.73 | 19.90 |

**Table 41 Effect of fox-LDL on cellular AChE activtity after 24 h treatment**

| Concentrations (μg/ml) | AChE activity (% of control) | | | | | | | | Mean | SEM |
|---|---|---|---|---|---|---|---|---|---|---|
| | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | No.7 | No.8 | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| Neo | 6.32 | 2.34 | 6.75 | 40.78 | 5.81 | 4.88 | 2.62 | 11.12 | 10.08 | 4.49 |
| 10 | 131.52 | 111.99 | 128.13 | 167.35 | 106.81 | 94.72 | 123.56 | 112.38 | 122.06 | 7.74 |
| 25 | 148.27 | 77.08 | 120.89 | 141.06 | 141.30 | 95.72 | 115.84 | 129.11 | 121.16 | 8.68 |
| 50 | 113.35 | 84.14 | 127.24 | 175.60 | 132.71 | 101.26 | 160.97 | 154.53 | 131.22 | 11.06 |
| 100 | 133.44 | 167.56 | 230.01 | 166.82 | 269.58 | 224.00 | 263.98 | 229.01 | 210.55 | 17.40 |
| 200 | 304.66 | 500.77 | 534.60 | 304.60 | 316.11 | 461.72 | 623.46 | 537.94 | 447.98 | 71.67 |

**Table 42 Effect of LDL and oxidized LDL on cellular AChE activtity in cell lysate**

| Concentrations | AChE activity (% of control) | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|
| | No.1 | No.2 | No.3 | No.4 | | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 | 0.00 |
| Neo | 7.69 | 10.17 | 9.86 | 3.11 | 7.71 | 3.26 | 1.88 |
| LDL | 66.72 | 102.43 | 102.65 | - | 90.60 | 20.68 | 14.62 |
| mox-LDL | 100.92 | 114.77 | 114.85 | 169.20 | 124.94 | 30.23 | 17.45 |
| fox-LDL | 126.34 | 156.30 | 149.63 | 177.66 | 152.48 | 21.13 | 12.20 |

**Note:** After treating cells with 100 µg/mL of LDL samples for 24 h, cells were lyzed and AChE activity measured

**Table 43 Effect of LDL and oxidized LDL on AChE expression**

| Treatments | AChE expression(% of control) | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|
| | No.1 | No.2 | No.3 | No.4 | | | |
| Control | 100.00 | 100.00 | 99.99 | 100.01 | 100.00 | 0.01 | 0.00 |
| LDL | 79.62 | - | 71.87 | 103.39 | 84.96 | 16.42 | 9.48 |
| mox-LDL | 86.86 | 95.96 | 131.96 | 106.63 | 105.35 | 19.49 | 11.25 |
| fox-LDL | 114.70 | 80.30 | 132.59 | - | 109.19 | 26.58 | 15.34 |

**Note:** After treating cells with 50 µg/mL of LDL samples for 24 h, Cell lysates from separated experiments were prepared and the AChE level was determined using anti-AChE (ab 31276) by Western blot

**Table 44 Effect of LDL and oxidized LDL on the release of APP into culture medium**

| Treatments | Bad density (% of control) | | | | | | | | | | Mean | SEM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | No.7 | No.8 | No.9 | No.10 | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| nLDL | 170.09 | 90.46 | 103.22 | 83.07 | 113.22 | 141.29 | 187.29 | 393.74 | 232.78 | 120.71 | 163.59 | 31.17 |
| mox-LDL | 156.76 | 102.59 | 135.30 | 76.32 | 62.80 | 81.40 | 381.33 | 382.90 | 103.37 | 65.23 | 154.80 | 41.15 |
| fox-LDL | 101.48 | 74.18 | 103.40 | 153.19 | 106.32 | 97.70 | 218.25 | 226.27 | 75.04 | 62.10 | 121.79 | 19.50 |

**Table 45 Effect of LDL and oxidized LDL on the level of sAPPα release into culture medium**

| Treatments | Babd density (% of control) | | | | | | | | | | Mean | SEM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | No.7 | No.8 | No.9 | No.10 | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| nLDL | 170.09 | 90.46 | 103.22 | 83.07 | 113.22 | 141.29 | 187.29 | 393.74 | 232.78 | 120.71 | 163.59 | 31.17 |
| mox-LDL | 156.76 | 102.59 | 135.30 | 76.32 | 62.80 | 81.40 | 381.33 | 382.90 | 103.37 | 65.23 | 154.80 | 41.15 |
| fox-LDL | 101.48 | 74.18 | 103.40 | 153.19 | 106.32 | 97.70 | 218.25 | 226.27 | 75.04 | 62.10 | 121.79 | 19.50 |

**Table 46 Effect of LDL and oxidized LDL on sAPPβ release in culture medium**

| Treatments | Babd density (% of control) | | | | | | | | | | Mean | SEM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | No.7 | No.8 | No.9 | No.10 | | |
| Control | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 |
| nLDL | 170.09 | 90.46 | 103.22 | 83.07 | 113.22 | 141.29 | 187.29 | 393.74 | 232.78 | 120.71 | 163.59 | 31.17 |
| mox-LDL | 156.76 | 102.59 | 135.30 | 76.32 | 62.80 | 81.40 | 381.33 | 382.90 | 103.37 | 65.23 | 154.80 | 41.15 |
| fox-LDL | 101.48 | 74.18 | 103.40 | 153.19 | 106.32 | 97.70 | 218.25 | 226.27 | 75.04 | 62.10 | 121.79 | 19.50 |

**Table 47 Effect of LDL and oxidized LDL on total of APP expression**

| Treatments | Total APP expression(% of control) | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|
| | No.1 | No.2 | No.3 | No.4 | | | |
| Control | 99.97 | 99.81 | 100.06 | - | 99.95 | 0.12 | 0.09 |
| LDL | 88.33 | 75.16 | 104.75 | - | 89.41 | 14.83 | 10.48 |
| mox-LDL | 104.75 | 85.22 | 104.01 | - | 97.99 | 11.07 | 7.83 |
| fox-LDL | 102.23 | 70.24 | 115.00 | - | 95.83 | 23.06 | 16.31 |

**Table 48 Effect of LDL and oxidized LDL on CREB activation in nuclear extraction after 15 min**

| Treatments | Density (% of control) | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|
| | N1 | N2 | N3 | N4 | | | |
| **Control** | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 | 0.00 |
| **nLDL** | 100.39 | 169.36 | 225.66 | 187.25 | 170.67 | 52.41 | 26.20 |
| **mox-LDL** | 155.51 | 284.87 | 166.34 | 86.80 | 173.38 | 82.25 | 41.12 |
| **fox-LDL** | 64.79 | 28.59 | 59.41 | 80.26 | 58.26 | 21.67 | 10.83 |

**Note:** After treating cells with 10 μg/mL of LDL samples for 15 min, Cell from separated experiments were extracted by nuclear extraction kit following the manufacturer were prepared and the CREB level was determined using anti-CREB by Western blot

**Table 49 Effect of LDL and oxidized LDL on CREB activation in nuclear extraction after 1 h**

| Treatments | Density (% of control) | | | | Mean | SD | SEM |
|---|---|---|---|---|---|---|---|
| | N1 | N2 | N3 | N4 | | | |
| **Control** | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0.00 | 0.00 |
| **nLDL** | 52.24 | 88.04 | 66.39 | 95.90 | 75.64 | 19.98 | 11.53 |
| **mox-LDL** | 138.32 | 110.28 | 185.62 | 96.96 | 132.80 | 39.21 | 22.64 |
| **fox-LDL** | 100.32 | 59.19 | 98.24 | 75.73 | 83.37 | 19.59 | 11.31 |

**Note:** After treating cells with 10 μg/mL of LDL samples for 60 min, Cell from separated experiments were extracted by nuclear extraction kit following the manufacturer were prepared and the CREB level was determined using anti-CREB by Western blot