ผลของไปเปอรีนต่อการตายของเซลล์จากไฮโดรเจนเปอร์ออกไซด์ใน เซลล์ประสาทเพาะเลี้ยง

นายประสิทธิ์ โอภาษ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชวิทยา ภาควิชาเภสัชวิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2549

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

EFFECTS OF PIPERINE ON HYDROGEN PEROXIDE – INDUCED CELL DEATH IN NEURONAL CELL LINE CULTURES

Mr Prasit Opas

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Pharmacy Program in Pharmacology

Department of Pharmacology

Faculty of Pharmaceutical Sciences

Chulalongkorn University

Academic Year 2006

Copyright of Chulalongkorn University

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Thesis Title	EFFECTS OF PIPERINE ON HYDROGEN PEROXIDE – INDUCED CELL DEATH IN NEURONAL CELL LINE CULTURES
By	Mr Prasit Opas
Field of Study	Pharmacology
Thesis Advisor	Assistant Professor Surachai Unchern, Ph. D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.... Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Pornpen Premyothin, Ph.D.)

THESIS COMMITTEE

....Chairman

(Associate Professor Siriporn Fungwitthaya)

....Thesis Advisor

(Assistant Professor Surachai Unchern, Ph.D.)

....Member

(Associate Professor Vimolmas Lipipun, Ph.D.)

....Member

(Associate Professor Mayuree Tantisira, Ph.D.)

ประสิทธิ์ โอภาษ : ผลของไปเปอรีนต่อการตายของเซลล์จากไฮโดรเจนเปอร์ออกไซด์ในเซลล์ประสาทเพาะเลี้ยง (EFFECTS OF PIPERINE ON HYDROGEN PEROXIDE-INDUCED CELL DEATH IN NEURONAL CELL LINE CULTURES) อ. ที่ปรึกษา : ผศ. ดร. สุรชัย อัญเชิญ, 76 หน้า.

การแพทย์พื้นบ้านใช้ไปเปอรีนอย่างแพร่หลายในการรักษาความผิดปกติต่างๆของระบบประสาทส่วนกลางและไปเปอรีนมีคุณสมบัติต้านออกซิเดชันชัดเจนซึ่งอาจชี้แนะศักยภาพในการบรรเทากระบวนการเสื่อมของประสาทอันเกิดจากความเครียดออกซิเดชัน ดังนั้นวัตถุประสงค์หลักของการวิจัยนี้คือศึกษาผลดีของไปเปอรีนต่อการตายของเซลล์ที่เกิดจากไฮโดรเจนเปอร์ออกไซด์ในเซลล์ประสาทเพาะเลี้ยง และค้นคว้ากลไกที่เป็นไปได้ของผลปกป้องเซลล์ประสาทดังกล่าว

การวิจัยครั้งนี้ใช้แบบจำลองระดับเซลล์ของการเสื่อมเซลล์ประสาทที่เกิดจากไฮโดรเจนเปอร์ออกไซด์ในเซลล์เพาะเลี้ยงของสายเซลล์ N1E-115 เป็นเครื่องมือศึกษาการตายของเซลล์ประสาทจากภาวะเครียดออกซิเดชันที่เกิดจากไฮโดรเจนเปอร์ออกไซด์ ใช้การวัด MTT reduction และ LDH release วิเคราะห์การอยู่รอดและการตายของเซลล์ ใช้การวัด lipid peroxidation ระดับ GSH ในเซลล์ และการตายแบบ apoptosis วิเคราะห์กลไกที่เป็นไปได้ของการปกป้องเซลล์ประสาท

ผลการทดลองของการศึกษาชี้แนะว่าไปเปอรีนมีผลต่อเซลล์ประสาทเพาะเลี้ยงในสองลักษณะ คือปกป้องเซลล์ที่ความเข้มข้นต่ำและเป็นพิษต่อเซลล์ที่ความเข้มข้นสูง การสัมผัสกับไปเปอรีนในความเข้มข้นที่ไม่เป็นพิษ (ช่วง 0.1-10 μM) พร้อมกับไฮโดรเจนเปอร์ออกไซด์ ปกป้องสายเซลล์เพาะเลี้ยง N1E-115 จากการบาดเจ็บและตายอันเกิดจากไฮโดรเจนเปอร์ออกไซด์ ในขณะที่การสัมผัสกับไปเปอรีนก่อนสัมผัสกับไฮโดรเจนเปอร์ออกไซด์ไม่แสดงผลดีแต่อย่างใด กลไกการออกฤทธิ์ของผลดีของไปเปอรีนต่อเซลล์ประสาทเพาะเลี้ยงยังไม่ทราบชัด แต่อาจเกี่ยวข้องกับการออกฤทธิ์โดยตรงและโดยอ้อมต่างๆ ของไปเปอรีน เช่น การต้านออกซิเดชันและการเก็บกวาดอนุมูลอิสระ การรบกวนปฏิกิริยา Fenton ซึ่งให้กำเนิด ROS การยับยั้ง lipid peroxidation ของเซลล์ การต่อต้านการตายแบบ apoptosis เป็นต้น ด้วยเหตุนี้จึงสมควรศึกษาต่อไปเกี่ยวกับคุณสมบัติทางเภสัชวิทยาโดยละเอียดและการประยุกต์ใช้เป็นเครื่องมือวิจัยของไปเปอรีน

ภาควิชา เภสัชวิทยา ลายมือชื่อนิสิต..

สาขาวิชา เภสัชวิทยา ลายมือชื่ออาจารย์ที่ปรึกษา..

ปีการศึกษา 2549

4676574133:MAJOR PHARMACOLOGY
KEYWORDS: PIPERINE / HYDROGEN PEROXIDE / N1E-115 CELL LINE / NEURONS / CELL DEATH

PRASIT OPAS: EFFECT OF PIPERINE ON HYDROGEN PEROXIDE-INDUCED CELL DEATH IN NEURONAL CELL LINE CULTURES. THESIS ADVISOR: ASST. PROF. SURACHAI UNCHERN, Ph.D., 76 pp.

Piperine has been widely used in folklore medicine to treat a variety of central nervous system disorders and has marked antioxidant property which may suggest its potential to alleviate oxidative stress-induced neurodegenerative process. The primary objectives of this study were to study beneficial effects of piperine on hydrogen peroxide (H_2O_2)-induced cell death in neuronal cell line cultures and investigate possible mechanisms underlying its neuroprotective effects.

A cellular model of neurodegeneration induced by H_2O_2 in N1E-115 cell line cultures was used to investigate the oxidative stress-induced neuronal cell death in this study. MTT reduction and LDH release assays were used to analyze cell survival and death. Lipid peroxidation, cellular GSH contents, and apoptotic cell death were used to analyze possible mechanisms of neuroprotection.

Experimental results from this study suggest that piperine possessed bimodal effects on neuronal cell cultures, cytoprotective at lower concentrations and cytotoxic at higher concentrations. Simultaneous exposure with nontoxic concentrations of piperine, in a range of 0.1-10 μM, may be protective against cell injury and apoptotic cell death from exposure to H_2O_2 in N1E-115 cell line cultures. However, pre-exposure to piperine did not show any beneficial effects. The mechanism of action underlying this beneficial effect of piperine on cultured neurons is unclear at the present time. Possibly, it may involve different direct and indirect actions of piperine, e.g., antioxidation and free radical scavenging, interference with Fenton's reaction which generates ROS, inhibition of cellular lipid peroxidation, counteraction to apoptotic cell death cascade. Further studies for clarifying detailed pharmacological properties and potential applications of piperine in experimental research are warranted.

Department of Pharmacology
Field of study Pharmacology
Academic year 2006

Student's signature...........................
Advisor's signature...........................

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my thesis advisor, Assistant Professor Dr. Surachai Unchern, for his guidance and encouragement of my research study.

Special thank are given to Department of Pharmacology and Biochemistry for provision of research equipments and facilities.

Also I wish to thank friends, staffs of the Department of Pharmacy and other people whose names have not mention for their assistance and great helpful support.

Finally, I especially wish to thank my family for their love, understanding, and moral support that inspire me to complete this work.

CONTENTS

		Page
ABSTRACT (THAI)		iv
ABSTRACT (ENGLISH)		v
ACKNOWLEDGEMENTS		vi
CONTENTS		vii
LIST OF TABLES		viii
LIST OF FIGURES		x
LIST OF ABBREVIATIONS		xii
CHAPTER		
I	INTRODUCTION	1
	Introduction	1
	Rationale of the study	2
	Hypothesis	3
	Objective	3
II	LITERATURE REVIEW	4
III	MATERIALS AND METHODS	23
IV	RESULTS	34
V	DISCUSSIONS AND CONCLUSION	47
REFERENCES		55
APPENDICES		64
BIOGRAPHY		76

LIST OF TABLES

Table Page

1. Effects of H_2O_2 on MTT reduction assay in Cultured
N1E-115 cell line (Incubation time = 1 hr).. 65

2. Effects of H_2O_2 on MTT reduction assay in Cultured
N1E-115 cell line (Incubation time = 3 hr).. 65

3. Effects of H_2O_2 on MTT reduction assay in Cultured
N1E-115 cell line (Incubation time = 6 hr)..................................... 66

4. Effects of H_2O_2 on MTT reduction assay in Cultured
N1E-115 cell line (Incubation time = 12 hr)...................................... 66

5. Effects of H_2O_2 on MTT reduction assay in Cultured
N1E-115 cell line (Incubation time = 24 hr)..................................... 67

6. Effects of H_2O_2 on LDH release assay in Cultured
N1E-115 cell line. (Incubation time = 6 hr).. 67

7. Effects of piperine on MTT reduction assay in Cultured
N1E-115 cell line (Incubation time = 1 hr).. 68

8. Effects of piperine on MTT reduction assay in Cultured
N1E-115 cell line (Incubation time = 3 hr)... 68

9. Effects of piperine on MTT reduction assay in Cultured
N1E-115 cell line (Incubation time = 6 hr)... 69

10. Effects of piperine on MTT reduction assay in Cultured
N1E-115 cell line (Incubation time = 12 hr)...................................... 69

11. Effects of piperine on MTT reduction assay in Cultured
N1E-115 cell line (Incubation time = 24 hr)...................................... 70

12. Effects of piperine on LDH release assay in Cultured
N1E-115 cell line. (Incubation time = 1 hr).. 70

13. Effects of piperine on LDH release assay in Cultured
N1E-115 cell line. (Incubation time = 3 hr).. 71

14. Effects of piperine on LDH release assay in Cultured
N1E-115 cell line. (Incubation time = 6 hr).. 71

15. Effects of piperine on LDH release assay in Cultured
N1E-115 cell line. (Incubation time = 12 hr)...................................... 72

16. Effects of piperine on LDH release assay in Cultured
N1E-115 cell line. (Incubation time = 24 hr)...................................... 72

17. Effects of piperine on H_2O_2 induced injuries in Cultured
N1E-115 cell line. Determine by MTT reduction assay (Cotreatment = 6 hr) 73

18. Effects of piperine on H_2O_2 induced injuries in Cultured
N1E-115 cell line. Determine by MTT reduction assay (Pretreatment = 12 hr) 73

19. Effects of piperine on H_2O_2 induced injuries in Cultured
N1E-115 cell line. Determine by MTT reduction assay (Pretreatment = 24 hr) 74

20. Effects of piperine on H_2O_2 induced injuries in Cultured
N1E-115 cell line. Determine by LDH release assay (Cotreatment = 6 hr) 74

21. Effects of piperine on H_2O_2 induced injuries in Cultured
N1E-115 cell line. Determine by LDH release assay (Pretreatment = 12 hr) 75

22. Effects of piperine on H_2O_2 induced injuries in Cultured
N1E-115 cell line. Determine by LDH release assay (Pretreatment = 24 hr) 75

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF FIGURES

Figure Page

1. Morphology of N1E-115 cell line …………………………………………… 4
2. Major molecular pathways leading to classical apoptosis………………… 8
3. Major molecular pathways leading to classical apoptosis-like and necrosis-likeProgrammed cell death………………………………………… 10
4. Chemical structure of piperine……………………………………………… 17
5. Effects of H_2O_2 on N1E-115 cell line cultures assessed by MTT reduction assay…………………………………………………….. 34
6. Effects of H_2O_2 on N1E-115 cell line cultures assessed by LDH release assay………………………………………………………… 35
7. Effects of piperine on N1E-115 cell line cultures assessed by MTT reduction assay…………………………………………………….. 36
8. Effects of piperine on N1E-115 cell line cultures assessed by LDH release assay……………………………………………………….. 37
9. Effects of piperine on toxicity induced by H_2O_2 in N1E-115 cell line cultures assessed by MTT reduction assay, (pretreatment = 12hr)…….. 38
10. Effects of piperine on toxicity induced by H_2O_2 in N1E-115 cell line cultures assessed by LDH release assay, (pretreatment = 12hr.)………… 39
11. Effects of piperine on toxicity induced by H_2O_2 in N1E-115 cell line cultures assessed by MTT reduction assay, (pretreatment = 24hr.) …… 40
12. Effects of piperine on toxicity induced by H_2O_2 in N1E-115 cell line cultures assessed by LDH release assay, (pretreatment = 24hr.)……….. 41

13. Effects of piperine on toxicity induced by H_2O_2 in N1E-115 cell line cultures assessed by MTT reduction assay, (cotreatment = 6hr.) ……….. 42

14. Effects of piperine on toxicity induced by H_2O_2 in N1E-115 cell line cultures assessed by LDH release assay, (cotreatment = 6hr.)…………. 43

15. Effects of piperine on H_2O_2-induced lipid peroxidation in N1E-115 cell line cultures……………………………………………………………………….. 44

16. Effects of piperine on H_2O_2-induced depletion of GSH level in N1E-115 cell line cultures………………………………………………………………………… 45

17. Effects of piperine on H_2O_2-induced apoptotic cell death in N1E-115 cell line cultures………………………………………………………………………… 46

LIST OF ABBREVIATIONS

β	=	beta
°C	=	degree celcius
μg	=	microgram
μl	=	microlitre
μM	=	micromolar
AA	=	arachidonic acid
Ca^{2+}	=	calcium ion
CNS	=	central nervous system
DMEM	=	Dulbecco's modified Eagle's medium
DMSO	=	dimethylsulfoxide
DNA	=	deoxyribonucleic acid
DNase	=	deoxyribonuclease
DPBS	=	Dulbecco's phosphate buffered saline
DPPH	=	1,1-diphenyl-2-picrylhydrazyl
DTNB	=	5,5'-dithiobis (2-nitrobenzoic acid)
ER	=	endoplasmic reticulum
et al.	=	et alii (and other)
FBS	=	fetal bovine serum
GSH	=	glutathione

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

GSSG = glutathione disulfide

IC_{50} = median inhibition concentration

K^+ = potassium ion

KCl = potassium chloride

kg = kilogram

L = litre

LDH = lactate dehydrogenase

MDA = malondialdehyde

mg = milligram

mM = millimolar

MTT = 3-(4,5 – dimethylthiazol – 2- yl) – 2,5 – diphenyltetrazolium bromide

mV = millivolt

Na^+ = sodium ion

NADP = nicotinamide adenine dinucleotide phosphate

NADPH = nicotinamide adenine dinucleotide phosphate (reduced form)

nm = nanometer

NMDA = N-methyl-D-aspartate

nmol = nanomole

NOS = nitric oxide synthase

PBS = phosphate buffered saline

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

RNA = ribonucleic acid

ROS = reactive oxygen speices

SEM = standard error of mean

SOD = superoxide dismutase

TBA = thiobarbituric acid

TBARS = thiobarbituric acid reactive substance

TNB = 5-thio-2-nitrobenzoate

CHAPTER I

INTRODUCTION

Oxidative stress, the exposure of cells to excessive reactive oxygen species (ROS), has been implicated as an etiologic factor in numerous diseases, including neurodegenerative diseases such as Alzheimer's disease (Richardson, 1993), Parkinson's disease (Olanow, 1992), amyotropic lateral sclerosis (Olanow, 1993), ischemia and excitotoxicity (Smith et al, 1991).

Hydrogen peroxide (H_2O_2) serves as a precursor for oxygen radicals within the cell. It is produced by peroxisomes and mitochondria and diffuses into neighboring cells through the extracellular space after a release from specialized cells during inflammation (Halliwell, 1992; Schreck and Bauerle, 1991). Cellular overload with H_2O_2 induces oxidative stress and may initiate a cascade of intracellular toxic events leading to energy failure, increased lipid peroxidation and subsequent cell death (Teepker et al, 2006).

The metal-dependent decomposition of H_2O_2 is a source of hydroxyl radical (^oOH), a species with a diffusion collision radius of 5–10 molecular diameters. The concept of a O_2^{o-} driven Fenton type reaction requires both O_2^{o-} and H_2O_2 as precursors of HO^o, it proceeds via an intermediate catalyst, such as a transition metal chelate (e.g., Fe^{3+}/Cu^{2+}), which is reduced by O_2^{o-} and reacts with H_2O_2 in a "Fenton–like reaction" to produce HO^o.

$$Fe^{3+} + O_2^{o-} \longrightarrow Fe^{2+} + O_2 \qquad (1)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO- + HO^o \qquad (2)$$

$$LH + HO^o \longrightarrow L^o + HOH \qquad (3)$$

$$L^o + O_2 + LH \longrightarrow LOOH + L^o \qquad (4)$$

Where LH and LOOH are membrane polyunsaturated fatty acid (PUFA) and PUFA hydroperoxide, respectively. These reactions lead to membrane damage and subsequent cell death (Ammad, 1995).

Piperine is an alkaloid found naturally in plants belonging to the Piperaceae family, such as *Piper nigrum*. L. (commonly known as black pepper), and *Piper longum*. L. (commonly known as long pepper). Black pepper and long pepper have been used in Ayurvedic medicine for treatment of various diseases. One such preparation is known by the Sanskrit name trikatu consists of black pepper and long pepper and ginger. Another preparation, known by the Sanskrit name pipali, consists of long pepper. It is thought that piperine is one of the major bioactive substances of these Ayurvedic remedies. Black pepper has also been used in traditional Chinese medicine to treat seizure disorders. A derivative of piperine, antiepilepsirine, was use in China to treat seizure disorders (Pei, 1983).

Piperine was shown to possess various pharmacological activities. In previous studies, piperine was shown to modulate the oxidative change by enhancing synthesis or transport of GSH in chemical carcinogen-induced oxidative stress in a rat intestinal model (Khajura et al, 1998), whereas Wonganan (2002) showed that piperine reversed glutathione diminution from LPS-induced toxicity. Piperine reduced high-fat diet-induced oxidative stress to the cell, (Vijakumar et al, 2004). On the other hand, the study by Karuppaiyah et al (2004) demonstrated that piperine significantly decreased the level of lipid peroxidation in similar to the study by Pensirinapa (2002) which showed significant beneficial effect of piperine on brain lipid peroxidation.

Therefore, available information reveals that piperine possesses marked antioxidant property which may suggest its potential to alleviate oxidative stress–induced neurodegenerative processes.

Rationale of the study

Piperine has been widely used in folklore medicine to treat a variety of central nervous system disorders and has marked antioxidant property which may suggest its potential to alleviate oxidative stress-induced neurodegenerative process. However, there has been a limited number of reported studies on piperine in respect to oxidative stress-induced neuronal cell death. Therefore, it is conceivable to investigate effects of piperine on H_2O_2-induced cell death in neuronal cell line cultures which could provide a clue and direction for further study in animal models of neurodegeneration.

Hypothesis

1. Piperine may protect neurons against cell death induced by hydrogen peroxide.
2. Neuroprotective effect of piperine against hydrogen peroxide-induced cytotoxicity may involve its antioxidant property.

Objectives of the study

1. To study effects of piperine on hydrogen peroxide-induced cell death in neuronal cell line cultures.
2. To investigate and suggest possible mechanisms underlying neuroprotective effects of piperine on hydrogen peroxide-induced cell death.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER II
LITERATURE REVIEW

N1E-115 cell line

N1E-115 murine neuroblastoma cell line was established in 1971 by Amano, Richelson, and Nirenberg by cloning the neuroblastoma tumor C-1300 that arose spontaneously in an A/J mouse. The clone N1E was subcloned by isolation of a single cell on glass shards. The adrenergic clone N1E-115 exhibits high levels of activity of tyrosine hydroxylase which is the rate limiting enzyme in the synthesis of catecholamines, and acetylcholinesterase which specifically degrades the neurotransmitter acetylcholine, however the clone is almost devoid of choline acetyltransferase, the enzyme that synthesizes acetylcholine.

N1E-115 cell line contains at least 14 receptors for neurotransmitters and related compounds including acetylcholine muscarinic m_1; acetylcholine muscarinic m_2; vasoactive intestinal peptide (VIP); adenosine; angiotensin II; bradykinin; enkephalin; glucagons; histamine H_1; 5-hydroxytryptamine (Serotonin, $5HT_3$); neurotensin; prostaglandin E; somatostatin; and thrombin. The cells have a reported doubling time of approximately 36 hr.

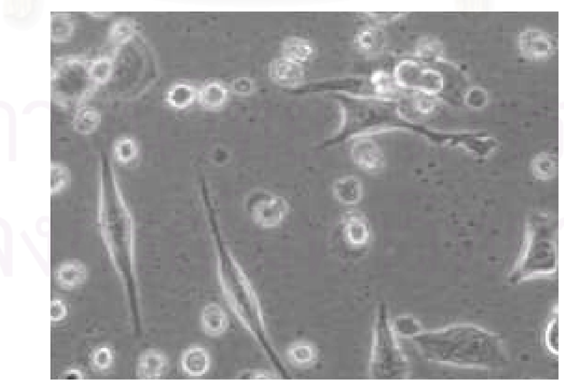

Figure. 1 Morphology of N1E-115 cell line. Photomicrograph was taken 12 hr after plating. Cells were seeded on multi-well plates and cultured in the presence of 10% FBS.

Hydrogen peroxide (H_2O_2)

The spontaneous or SOD–catalyzed imbalance of O_2^{o-} yields H_2O_2, a species which is generated directly via two electron transfers by several oxidases. H_2O_2 is not a free radical by itself. However, its chemical reactivity is substantially enhanced by two features of H_2O_2. First, and at a variance with its precursor O_2^{o-}, H_2O_2 can freely cross biological membranes, a property apparently shared by the conjugate acid of O_2^{o-}, HO_2^{o}. Second, H_2O_2 is required for the formation of more potent oxidants such as the hydroxyl radical (HO^{o}) and oxoferryl complexes, upon its reaction with metal chelates and hemoproteins, respectively (Ahmad, 1995).

The steady state concentration of H_2O_2 in liver originating from various sources was estimated about 10^{-9} – 10^{-7} M and that of O_2^{o-}about 10^{-11} M. This figure for H_2O_2 steady state concentration is expected to be higher in organs with less effective mechanisms than liver for disposal of H_2O_2. Thus, in most normal cells rate of the steady state concentration of H_2O_2 and O_2^{o-} is represented by $[H_2O_2]/[O_2^{o-}]$ about 10^3 (Ahmad, 1995)

Programmed cell death (PCD)

PCD is generally defined as a series of stereotypical biochemical and morphological steps leading to cell demise ((Lossi and Merighi, 2003; Krantic et al, 2007). As opposed to cell death by necrosis, PCD is an active process by which dying cells are removed in a safe, non-inflammatory manner. As such, the term programmed stresses that this cell death modality has an intrinsic, genetically defined character regulated by various signaling pathways and that it is clearly distinct from the accidental induction of necrosis by cell damage or "bioenergetic catastrophe". Furthermore, PCD is a fundamental physiological phenomenon, as it is involved in controlling the balance between proliferation and differentiation during development (e.g., mammalian organogenesis) and in the optimization of cell/tissue functions throughout adulthood (e.g., thymic maturation of T-lymphocytes). Recently, PCD has also been associated with pathological processes such as cancer and neurodegeneration.

Classification of PCD

The existence of multiple types of PCD is well documented, but difficulties in reaching a consensus on the criteria used to distinguish them have hampered efforts to establish a universal classification. As mentioned above, the programmed character is clearly a PCD hallmark, but it is often employed to refer to particular aspects of the phenomenon. For instance, it is used to indicate the developmentally programmed occurrence of PCD, or to reflect the programmed stereotypical succession of morphological and biochemical events in non-developmental PCDs. However, the term programmed is generally employed as a synonym of regulated, leading to a broad classification of PCDs into apoptosis, necrosis and autophagy. One of the more restraining classifications, based on the criterion of nuclear morphology, divides PCDs into classical apoptosis, apoptosis-like PCD and necrosis-like PCD (Figure 2). These are, respectively, characterized by "crescent-like" (type 2), partial/peripheral (type 1) or absent nuclear chromatin condensation. The other common criteria used for classification are biochemical (based on the activation of a specific class of proteases) and pharmacological (based on the capacity of specific protease inhibitors to block a given type of PCD). Experimentally, however, these parameters have only been well documented for classical apoptosis, currently leaving nuclear morphology as the most accurate criterion. This might soon change, as our understanding of apoptosis-like PCD is rapidly evolving despite the absence of selective pharmacological inhibitors of the proteases involved. In contrast, our knowledge of necrosis-like PCDs remains very limited.

Mechanisms of apoptosis

Classical apoptosis, the best known phenotypic expression of PCD, consists of at least two phases, initiation and execution, resulting from the activation of cysteine-dependent, aspartate-directed proteases termed *caspases*. The death receptors (extrinsic) and mitochondrial (intrinsic) pathways represent the canonical routes of caspase activation during the initiation phase. The death receptors-mediated recruitment of procaspases- 8 or procaspases-2/-10, as well as the mitochondrial pathway-induced

activation of caspase-9 via cytochrome *c* release, both converge to the activation of procaspase-3 and of caspases-6 and -7, further downstream. These pathways are all associated with caspase activated DNase (CAD) activation, leading to “typical” inter-nucleosomal DNA fragmentation (Figure 2).

Death receptors (DR) are activated by extracellular ligands such as tumor necrosis factor-α (TNF-α), fas ligand (FasL, also called CD95L/Apo1L), TNF-like WEAK inducer of apoptosis (TWEAK) and TNF-related apoptosis-inducing ligand (TRAIL). Their cognate receptors belong to the TNF receptor (-R) super-family. These receptors include Fas/CD95, which binds FasL/CD95L, TNF-R interacting with TNF and lymphotoxin, TRAMP (also called Apo3DR3/WSL-1/LARD) which binds TWEAK. Fibroblast growth factor-inducible 14 (Fn14) binds TRAIL as do DeCoy receptors (DCRs)1/2. Osteoprotegerin (OPG) acts as a soluble antagonist receptor with a capability to inhibit TRAIL-mediated killing. These receptors contain death domains (DD) and death effector domains (DED) capable of homeotypic protein-protein interactions. Receptor assembly into oligomers is triggered by ligand binding, resulting in the formation of a death-inducing signaling complex (DISC) through conformational alterations. Adaptor proteins (e.g., TRADD, RaiDD, FADD) bearing both DD and DED domains are then recruited to the relevant receptors, that further recruit procaspase-8, which is subsequently autoproteolytically activated with the local accumulation of its pro-enzyme in the vicinity of DISC.

Considerable evidence suggests that the initiation of intrinsic apoptotic pathway takes place in mitochondrial membranes, although it is now increasingly clear that this pathway can also be triggered at the level of other cellular organelles such as the endoplasmic reticulum, nucleus and lysosomes. However, the involvement of these other organelles requires that extra-mitochondrial triggers first converge towards mitochondria. The intrinsic pathway is initiated by mitochondrial outer membrane permeabilization (MOMP) and the subsequent release of toxic mitochondrial proteins into the cytoplasm. Among these, cytochrome c was the first demonstrated trigger for apoptosome formation. More recently, other mitochondrial proteins such as second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI (Smac/DIABLO) and

serine protease high temperature requirement protein A2 (HtrA2/OMI) have also been involved in caspase activation. However, in contrast to cytochrome *c* which initiates apoptosome formation by promoting the interaction between apoptotic protease-activating factor 1 (Apaf-1) and procaspase-9 and further activation of caspase-3 and caspase- 6/-7, Smac/DIABLO and HtrA2/OMI participate indirectly to the activation of the cascade. Indeed, Smac/DIABLO and HtrA2/OMI antagonize inhibitors of apoptosis proteins (IAPs) and thus release the blockage exerted by these caspase inhibitors. This blockage consists in preventing caspase-9 dimerization and inhibiting the enzymatic activity of caspases-3 and -7. Once fully activated, these caspases cleave multiple cellular substrates, including inhibitor of CAD (ICAD) leading to DNA fragmentation following the nuclear translocation of CAD.

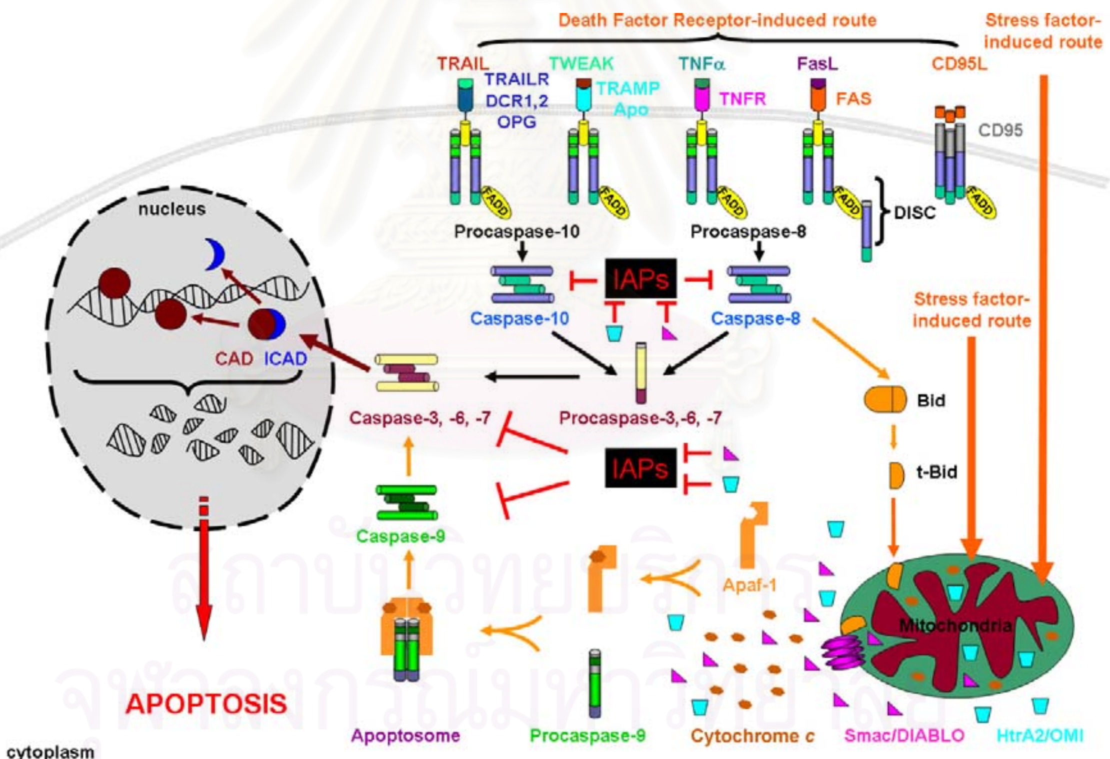

Figure 2. Major molecular pathways leading to classical apoptosis: The two major signaling pathways leading to classical apoptosis involve caspase-activated cascades ultimately leading to the activation of executive caspases, such as caspases-3, -6 and -7. Depending on the type of stimuli and/or cell type involved, the apoptotic response is set in motion by an “extrinsic” pathway following the activation of caspase-8 or -10 by

the activation of a death receptor, or by an "intrinsic" pathway involving apoptogenic mitochondrial proteins such as cytochrome c. Along the "extrinsic" pathway, the activation of "death receptors" (e.g., TRAILR, TRAMP, TNFR, FAS, CD95) by their cognate ligands (TRAIL, TWEAK, TNFα, FasL, CD95L, respectively) triggers the association of adaptor proteins containing death domains (for the sake of clarity, only FADD is depicted here). Recruitment of FADD is then followed by the recruitment of the inactive procaspase-10 (or -8 in some cellular contexts). Theses procaspases are subsequently activated in an auto-proteolytical manner due to the local accumulation of the relevant pro-enzymes in the vicinity of DISC. The activation of caspase-10 or -8 initiates the caspase cascade leading ultimately to the activation of the caspase-3 and final "execution" phases of cell death which is shared with the "extrinsic" pathway. Along the "extrinsic" pathway, activated caspase-8 catalyzes the cleavage of the pro-apoptotic protein Bid yielding tBid. The latter further translocates to the outer mitochondrial membrane where it participates in pore formation. This allows the release of the mitochondrial proteins such as cytochrome *c*, Smac/DIABLO, HtrA2/OMI. Cytochrome *c* subsequently associates with procaspase-9 and the adaptor protein Apaf-1 to form a complex called apoptosome. This complex gives rise to an active caspase-9, which in turn activates executive caspases. Smac/DIABLO and HtrA2/OMI act as the indirect activators of caspases since they block the inhibition exerted by IAPs upon these proteases. Activated "executioner" caspases (mainly caspase-3) mediate the release of CAD from the complex with its endogenous inhibitor ICAD through a proteolytic cleavage. Activated CAD then brings about the internucleosomal lysis of DNA yielding DNA fragments, which are multiples of 180–200 bp.

Apoptosis-like and necrosis-like PCDs

Apoptosis-like and necrosis-like PCDs are often considered as alternative death programs, mainly because of their independence from caspases (Figure 3). Indeed, it is assumed that these PCDs can still occur when caspases are inhibited. Caspase inhibition can result, among other factors, from energy depletion, a mutation in the Apaf-1 gene and nitrative/oxidative stress. Neurons are particularly sensitive to both energy depletion and oxidative stress. They depend entirely on the aerobic metabolism of

glucose, which generates ATP and ROS as by-products of incomplete oxygen reduction to water in the course of oxidative phosphorylation.

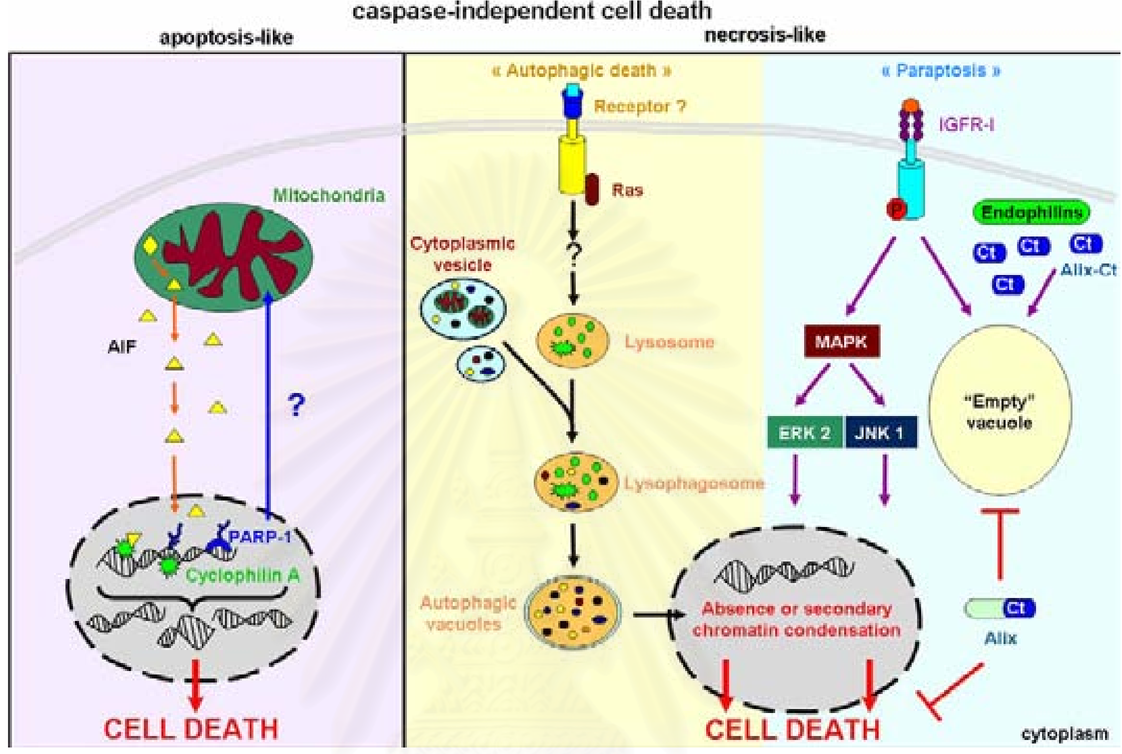

Figure 3. Major molecular pathways leading to apoptosis-like and necrosis-like programmed cell deaths (PCD): Apoptosis-like PCD is mediated by mitochondrial effectors, such as AIF. When apoptosis is induced through this pathway, PARP-1-mediated DNA damage is signaled to mitochondria via an unknown mechanism. This event leads to AIF release and translocation from mitochondria to the nucleus, leading to chromatin condensation and large-scale DNA fragmentation (>50 kbp). AIF itself does not have an endonuclease activity. However, it has been shown to enhance endonuclease activity through its cooperation with endonuclease G in *C. elegans* and cyclophilin A in mammalian cells. Necrosis-like PCDs involve different pathways resulting in absent or secondary chromatin condensation. Although these pathways remain to be fully understood, some of their key components have emerged from recent investigations. Thus, autophagic death is thought to be triggered by Ras, which would lead, directly or indirectly to the formation of lysophagosomes and autophagic vacuoles. Paraptosis, which involves extensive cytoplasmic vacuolation, has been described to

occur following the activation of IGFR-I, NK-1 or EGFR. The involvement of Alix and of the MAPK pathway have also been involved in this PCD, although the latter might be specific to certain cell types.

Considerable progress has recently been made in understanding the molecular mechanisms involved in apoptosis-like PCD, whereas much less is known about necrosis-like PCDs such as deaths involving autophagy and paraptosis. It has been reported that Ras can trigger autophagic PCD. However, it appears clearly that in addition to its involvement in a particular form of PCD (characterized morphologically by the appearance of autophagic, double membraned vacuoles) autophagy plays also role in cell survival. It thus mediates a cytoprotective response in adverse conditions (e.g., low-nutrient states, pathological conditions) by catabolizing intracellular substrates to produce energy, and by removing damaged organelles such as mitochondria to prevent apoptosis. Therefore, although autophagy by itself does not induce cell death, it is associated with PCD in particular conditions (e.g., when pro-apoptotic proteins Bax and Bak are down-regulated). The criteria for identifying this type of PCD were exclusively morphological until recently. However, identification of genes such as Atg5 or Becn 1, and the fact that their inactivation protects from "autophagic" PCD, strengthened the experimental evidence in favor of the existence of this PCD as a specific (i.e., different from known PCDs) cell death modality.

Engagement of several types of receptors by their cognate ligands has been reported to trigger paraptosis. They involve insulin-like growth factor IGFR-I (IGF receptor-I) interaction with IGF-I, neurokinin-1 receptor activation by substance P and epidermal growth factor (EGF)/EGF receptor (EGFR) interaction. In addition, overexpression of TAJ/TROY members of the TNF receptor superfamily leads to non-apoptotic cell death with paraptosis morphology in transfected cells. Similarly, human glioma cells retrovirally transduced with a gene encoding the membrane form of macrophage colony-stimulating factor (mM-CSF) acquire the capacity to die by a process resembling paraptosis when exposed to monocytes/macrophages in cytotoxicity assays. Primary neurons also display the capacity to die by this type of PCD. However, it should be stressed that in all these cases, paraptosis has been defined

principally according to morphological criteria, including extensive cytoplasmic vacuolation resulting from progressive swelling of mitochondria and endoplasmic reticulum. In addition, this PCD modality has been associated with loss of the mitochondrial transmembrane potential and phosphatidylserine externalization at least in some instances. It has further been shown that paraptosis may involve the activation of MAP kinases although this signaling pathway might be specific to certain cell types since it is not obviously activated during the initiation of this program. However, in all cases reported thus far, paraptosis is not affected by broad-range caspase. Interestingly, the ability of AIP-1/Alix to block paraptosis (which can be achieved by its over-expression) has recently been described as a hallmark of this PCD modality.

Specificity of neuronal PCDs

The importance of classical apoptosis as a critical form of PCD in the adult nervous system is still debated. According to some studies, apoptosis is down-regulated (at least under physiological conditions) due to a differentiation-associated reduction in Apaf-1 expression and increased efficacy of IAPs to stringently regulate it. Moreover, the over-expression of IAPs confers neuroprotection *in vivo*, whereas neuronal IAP (NAIP)-deleted mice display increased vulnerability to kainic acid injury. The precise types of neuronal death associated with "normal" physiological brain aging are currently unknown. The lack of knowledge in this field is likely to be one of the reasons behind the present confusion concerning the operational modalities of neuronal death under "physiological conditions". Indeed, classical apoptosis may be the predominant neuronal death phenotype occurring during synaptogenesis.

Accumulated evidence suggests that under pathological conditions, during both acute and chronic neurodegenerations, all types of PCD phenotypes can occur. Importantly, and under some circumstances, neuronal death is intimately linked to an unscheduled tentative re-entry into cell division cycle. Adult neurons are terminally differentiated cells that are excluded from the cell cycle (G0 quiescent state). Tentative re-entry into the cell cycle by forced expression of oncogenes, for example, triggers cell death rather than uncontrolled proliferation. It has also been shown that adult neurons

can respond to growth factors by reinitiating the cell cycle, without the ability to progress beyond cyclin B1 expression, suggesting that cell death occurs at the G2/M transition. Similarly, cortical neurons treated in vitro with amyloid beta (Aβ; a cleavage product of the amyloid precursor protein, APP) re-enter the cell cycle, pass through the G1/S transition and probably die at, or just before the G2/M transition. It is currently unclear why in other paradigms of neurodegeneration such as ischemia and kainic acid-induced seizures, neurons die at the G1/S transition or at the beginning of the S-phase, after DNA replication; hypotheses respectively, based on cyclin D1 induction and increased BrdU incorporation. It should be stressed that conclusions about re-entry of dying neurons into the cell division cycle have been mainly based on the observed increase in BrdU incorporation. However, it has been recently shown in neurons that such an increase might rather reflect cell death.

Molecular mechanisms linking aberrant cell cycle re-entry and cell death remain to be explored. However, it has been proposed that oxidative stress might be associated with neuronal cell death, although it is not yet clear whether it represents the cause or the consequence of death. Accordingly, the putative involvement of AIF in neuronal PCD induction is a particularly exciting research field.

In neurodegenerative disorders and traumatic neuronal injury, neuronal loss may be linked to apoptosis. However, in these conditions cell death may also be necrotic. In the latter (exogenous cell death), alterations of the cellular environment eventually result in cell swelling and disruption of the cell membrane, while the main elements of the apoptotic machinery are constitutively expressed or generated by the cell itself (cell suicide). Although the two types of cell death have initially been considered independent from each other, it is now clearly emerging that they share some cellular and molecular features, and that cells can switch from one mode of death to the other upon different conditions. A tremendous amount of literature in the recent years has deepened our knowledge on the cellular and molecular mechanisms of apoptosis in neurons and other cells.

H_2O_2-induced apoptotic cell death

H_2O_2, a byproduct of oxidative stress, has been implicated to trigger apoptosis in neurons leading to major neurodegenerative diseases. H_2O_2-induce apoptotic cell death was reported in several cell types, for examples, rat glioma cells C6 (Chen et al, 2006), human leukemia HL-60 cell line (Lennon et al, 1991). *In vitro* model of oxidant-induced neuronal injury has been reported in PC12 cell cultures in which H_2O_2 induced membrane blebling and microtubule destruction (Hinshaw, 1993). Exposure to H_2O_2 induced cell death via apoptosis in cultured cortical neurons within 3 hr, as assessed by cell viability, morphological and ultrastructural measures (Whittemore et al, 1994). In addition, exposure to high concentrations of H_2O_2 (100 μM) caused an increase in intracellular free calcium within 3 hr. (Whittemore et al, 1995), whereas sodium nitroprusside induced apoptotic cell death via production of H_2O_2 in murine neuroblastoma N1E-115 cell (Yamada et al, 1996).

Other actions of H_2O_2

H_2O_2 incubation decreased ATP-levels in a dose- and time-dependent manner in all neuronal cell systems tested. Such effects were most pronounced in primary hippocampal neurons. In cortical cells, increased ATP-levels were notable under low H_2O_2 concentration. A dose-dependent decrease in ATP-concentration was observed after treatment with Ca^{2+}, which was further enhanced by additional H_2O_2 challenge (Teepker et al, 2007). Reactive species could induce different cell death mechanisms in cultured neurons. H_2O_2 led to a necrosis-like cell death that did not induce caspase activation, phosphatidylserine translocation, or changes in calcium levels. Incubation of copper/zinc superoxide dismutase (Cu/Zn-SOD) or manganese superoxide dismutase (Mn-SOD) together with H_2O_2 caused rapid breakdown of nitric oxide (NO) and production of peroxynitrite ($ONOO^-$) indicated by the oxidation of dihydrorhodamine-1,2,3 to rhodamine-1,2,3. H_2O_2 addition to macrophages (cell line 5774) expressing the inducible form of NO-synthase (i-NOS) caused rapid breakdown of NO that they produced and this was followed by subsequent $ONOO^-$ production in the macrophages. Exposure of cells to 200 μM H_2O_2 caused a rapid increase in HSF-1 DNA binding that

was evident within 10 min and caused a robust increase that reached 8-fold levels of the basal activity. In comparison, the transcription factors, activator protein-1 (AP-1) and early growth response-1 (EGR-1), were activated more slowly and to a lesser extent. Activation of HSF-1 was associated with a cytosol-to-nuclear translocation of HSF-1 protein, and was detected at concentrations of H_2O_2 of 100 μM and greater. Intracellular glutathione modulated H_2O_2-induced HSF-1 DNA binding activity, as depletion of glutathione caused HSF-1 to be activated at lower concentrations of H_2O_2 (25 μM) and supplement of glutathione blocked HSF-1 activation by 100 to 400 μM H_2O_2. Cybrid cells (SH-SY5Y cells) in which mitochondria were replaced with platelet mitochondria from Alzheimer's disease (AD) patients or matched control subjects were used to test effects of chronic oxidative stress caused by excessive production of reactive oxygen intermediates (ROIs) on HSF-1 activity. Basal and maximal (induced by H_2O_2 in glutathione depleted cells) HSF-1 DNA binding activity were lower in AD than control cybrids. (Bijur et al, 1999)

Enzymatic removal of H_2O_2

Catalase (CAT)

Catalase acts sequentially to SOD to dismutase two identical substrate molecules to lower the oxidation state. This overall reaction proceeds in two steps.

$H_2O_2 + H_2O_2 \longrightarrow 2H_2O + O_2$ (1)

$CAT\text{-}Fe^{3+} + H_2O_2 \longrightarrow$ Complex I (2)

Complex I $+ H_2O_2 \longrightarrow CAT\text{-}Fe^{3+} + 2H_2O + O_2$ (3)

Complex I +DH2 $\longrightarrow CAT\text{-}Fe^{3+} + 2H_2O + D$ (4) (Ahmad, 1995)

Ascorbate peroxidase

Ascorbate peroxidase (AP) removes H_2O_2 at the expense of ascorbate (AH2) which is reduced to dehydroascorbate (DHA)

$H_2O_2 + AH2 \longrightarrow 2H_2O + DHA$ (Ahmad, 1995)

Cytochrome-c peroxidase

In fungi, cytochrome-c peroxidase (CCP) is the main H_2O_2 and other peroxide scavenging peroxidase. The reaction catalyzed is as follows

H_2O_2 + Ferrocytochrome-c $\rightarrow$ $2H_2O$ + 2Ferricytochrome-c (Ahmad, 1995)

Glutathione peroxidases

A cytosolic Sc-dependent glutathione peroxidase (GPOX) presenting in the cell cytosol and mitochondrial matrix has long been regarded as a crucial enzyme for the removal of cytotoxic hydroperoxides. In vertebrate species, this enzyme has an absolute requirement for the hydrogen donor GSH for catalytic activity. In the reaction catalyzed, the enzyme reduces H_2O_2 to two molecules of H_2O, or reduces membrane peroxides (ROOHs/LOOHs) to one molecule of H_2O and one molecule of the corresponding innocuous alcohol.

H_2O_2 + 2GSH $\rightarrow$ $2H_2O$ + GSSG (1)

LOOH + 2GSH $\rightarrow$ H_2O + LOH + GSSG (2) (Ahmad, 1995)

Glutathione reductase

In all aerobic organisms, the redox status of cells heavily relies on the maintenance of glutathione in its reduced state, GSH, and prevention of its oxidation to the oxidized state, GSSG.

GSSG + NADPH + H^+ $\rightarrow$ 2GSH + NAD^+(P), (Ahmad, 1995)

Piperine

Pepper is one of the oldest and most important member of the spices. It was used by the ancient Greeks and was highly valued by the Romans. The search for a trade route by sea to India was to some extent instigated by the European demand for pepper which, during the Middle Ages, was of great economic importance in Western

Europe. The two forms of the spice, black pepper and white pepper, are obtained from the fruits and seeds of *Piper nigrum*, black pepper consisting of the dried ground fruits (peppercorn) and white pepper consisting of the dried ground seeds. They are still used extensively as a condiment and flavoring for all types of savory dishes, for preserving and pickling, and in the manufacture of sauces, ketchups, and brandy (Govindarajan, 1977). Among the *Piper* species, black pepper (*Piper nigrum* Linn.) and long pepper (*Piper longum* Linn.) are commonly used in many regions of the world. The pungency of pepper is due to the presence in the fruit of various resins and a yellow crystalline alkaloid, piperine, which is present to the extent of 4.5-8%.

Piperine is a solid substance practically insoluble in water. It is a weak base that is tasteless at first, but leaves a burning aftertaste. Piperine belongs to the vanilloid family of compounds, a family that also includes capsaicin, the pungent substance in hot chili. Its molecular formula is $C_{17}H_{19}NO_3$, and its molecular weight is 285.34 daltons. Piperine is the trans-trans stereoisomer of 1-piperoylpiperidine. It is also known as (E,E)-1-piperoylpiperidine and (E,E)-1[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentdionyl]piperi-dine.

Figure 4: Chemical structure of piperine

Biological activities of piperine

Several studies on the biological activity of piperine indicated a wide variety of effects on several systems, e.g., central nervous system (CNS), cardiovascular system, respiratory system, and hepatic drug metabolism (Atal et al, 1985).

Pharmacological effects of piperine on the central nervous system

Piperine administration had beneficial effects on transient cerebral ischemia (TCI)-induced cognitive deficit and brain lipid peroxidation increase in mice (Pensirinapa, 2002). The close correlation between effects of piperine on both indications of brain injury also implied that the attenuation of TCI-induced cognitive deficit may involve, at least partly, the antioxidant property of piperine. Piperine, especially at low concentrations, might have stimulatory effect on glial cell metabolic activity and facilitate glial cell functions in brain inflammatory responses (Wonganan, 2002). Antiepilepsirine, a derivative of piperine, was used as antiepileptic drug in treating different types of epilepsy in China (Pei, 1983). On the other hand, some studies reported that piperine, especially at high doses, has respiratory stimulant and convulsant properties in various laboratory animals (Kulshrestha et al, 1969, Singh et al, 1973). Pharmacological studies indicated that piperine and several of its derivatives protected rat and mice against various kinds of experimental convulsions, including those induced by maximal electroshock, picrotoxin and strychnine (Pei, 1979). Recent research suggested that piperine significantly blocked convulsions induced by intracerebro-ventricular injection of kainate but had no or only slight effect on convulsions induced by L-glutamate and N-methyl–D-aspartate (NMDA). Although piperine did block convulsions induced by kainate, the compound did not appear to act as a kainate receptor antagonist (D'Hooge et al, 1996).

Hepatoprotective effect of piperine

Piperine did not act as a hepatotoxic agent but functioned as a chemopreventive substance by enzyme modulation. The compound was found to exert significant protection against chemically induced hepatotoxicity by reducing both *in vitro* and *in vivo* lipid peroxidation, enzymatic leakage of glutamate-pyruvate transaminase (GPT) and alkaline phosphatase (AP), and by preventing the depletion of reduced glutathione and total thiols in the intoxicated mice (Koul and kapil, 1993)

Antiinflammatory effect of piperine

Piperine treatment significantly reduced the proinflammatory cytokines, as considered by the expression of IL-1β, IL-6, TNF-α, GM-CSF and IL-12. Piperine at concentrations of 2.5, 5, and 10 μg/ml inhibited the collagen matrix invasion of B16F-10 melanoma cells in a dose-dependent manner. Piperine could inhibit the matrix metalloproteinase production which was demonstrated by zymographic analysis. Nuclear translocation of p65, p50, c-Rcl subunits of NF-κB and other transcription factors, such as ATF-2, c-Fos and CREB, were inhibited by treatment with piperine (Pradeep et al, 2004). Moreover, piperine showed anti-inflammatory activity in animal models of inflammation (Lee et al, 1984; Mujumdar et al, 1990a; Dhuley et al, 1993).

Antioxidative effect of piperine

Administration of piperine significantly decreased levels of lipid peroxidation, protein carbonyls, nucleic acid content and polyamine synthesis that were found to be increased in lung cancer bearing animals, (Selvendiran et al, 2004). Carcinogen treatment induced GSH depletion with substantial increase in thiobarbituric reactive substances (TBARS) and enzyme activities while piperine treatment with carcinogen resulted in inhibition of TBARS. It mediated a significant increase in GSH levels and restoration in γ-GT and Na^{+}-K^{+}-ATPase activities (Khajuria et al, 1998). Significantly elevated levels of TBARS, conjugated dienes (CD), and significantly lower activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-s-transferase (GST) and reduced glutathione (GSH) in the liver, heart, kidney, intestine and aorta were observed in rats fed the high fat diet as compared to the control rats. Simultaneous supplementation with black pepper or piperine (0.02 g/kg body weight) lowered TBARS and CD levels and maintained SOD, CAT, GPx, GST, and GSH levels to near those levels of control rats (Vijayakumar et al, 2004). However, many studies indicated that piperine contains weak antioxidant and free-radical scavenging activities comparing to other spice principles such as curcumin from turmeric, capsaicin from red chillies, and eugenol from cloves (Krishnakantha and Lokesh, 1993)

Other pharmacological activities of piperine

Supplementation with beta carotene plus piperine for 14 days produced a 60% greater increase in area under the serum beta-carotene curve (AUC) than that observed during supplementation with beta-carotene plus placebo (Badmaev et al, 1999). In addition, supplementation of 120 mg of coenzyme Q_{10} with piperine for 21 days produced a significant, approximately 30%, greater AUC than that observed during supplementation with coenzyme Q_{10} plus placebo (Vladimir et al, 2000). Piperine has been found to inhibit human P-glycoprotein and CYP-3A4 enzymes important for the metabolism and transport of xenobiotics and metabolites. Some the claim that piperine may aid in the digestion of food is based on some experimental animal data showing that dietary piperine seems to enhance pancreatic amylase, lipase, tripsin, and chymotrypsin activity. Administration of alcoholic extract of *Piper longum* (10 mg/dose/animal) as well as piperine (1.14 mg/dose/animal) could inhibit the solid tumor development in mice induced with DLA cells and increase the life span of mice bearing enrich ascites carcinoma tumor. Administration of *piper longum* extract and piperine increased the total WBC count in Balb/c mice. The number of plaque forming cells also enhanced significantly by administration of the extract and piperine on 5th day after immunization. Bone marrow cellularity and α-esterase positive cells were also increased by the administration of *Piper longum* extract and piperine (Sunila and Kuttan, 2004). Some studies reported antiamebic, antipyretic, and analgesic activities of piperine (Lee et al., 1984).

Toxicological effects of Piperine

Piperine had shown some evidence of being mutagenic and potentially carcinogenic under some circumstances. It gave rise to mutagenic products on reaction with nitrites. This causes concern since nitrites and piperine may be consumed simultaneously. Risk might increase with high dose piperine supplementation. Piperine appeared to enhance the bioavailability of aflatoxin B1 in rat tissues and piperine was found to be cytotoxic to cultured brain neurons. In a recent study utilizing albino rats, piperine, given at dose of 5 and 10 mg/kg body weight for 30 days, resulted in significant

reduction in the weights of tests and accessory sex organs as well as severe damage to seminiferous tubules (Malini et al, 1999). The 5 mg/kg dose resulted in partial degeneration of germ, decrease mating performance, decrease fertility and anti-implantation activity, along with some other adverse reproduction events were observed in mice given very high dose of piperine.

Acute toxicity of piperine was investigated in different species of animals including mouse, rat and hamster. After receiving a fatal dose of piperine, they immediately exhibited an increase in locomotor activity. This activity was progressively increased from running, jumping, to convulsion and finally the death was occurred with whole body muscle spasm. The cause of death may possibly be the certain types of neurotoxicity and respiratory paralysis. It was reported that the LD_{50} values for single intravenous and intragastric administration of piperine are 15.1 and 330 mg/kg body weight respectively (Piyachaturawat et al, 1983).

Moreover, piperine pretreatment in rats potentiated carbon tetrachloride (CCl_4)-induced hepatotoxicity by interacting with liver cells and accelerated biotransformation of CCl_4, thereby increasing lipid peroxidation and enhancing hepatotoxicity (Piyachaturawat et al, 1995). *In vitro* studies demonstrated the cytotoxic effect of piperine on embryonic rat brain neurons in culture (Unchern et al, 1994a). It was shown to suppress both neuronal survival and neurite extension of viable neurons (Unchern et al, 1994b). In this connection, piperine-induced cytotoxicity was relatively selective for cultured neurons in comparison with cultured astrocytes (Unchern et al, 1997). Moreover, death of cerebellar granule neurons induced by piperine is distinct from that induced by low potassium medium (Unchern et al, 1998).

Effects of piperine on drug metabolism

Piperine increased serum levels and lengthened serum half lives of some drugs, such as propranolol and theophylline (Bano et al, 1991). The mechanism might be an inhibition of certain enzymes involved in the biotransformation of affected drugs. Piperine had been found to be a nonspecific inhibitor of drug and xenobiotic metabolism. It appeared to inhibit many different cytochrome P450 isoforms, as well as UDP-

glucuronyltransferase, hepatic arylhydrocarbon hydroxylase, and other enzymes involved in drug and xenobiotic metabolism. Other mechanisms for piperine-induced increased bioavailability of drugs and nutrients might involve non-specific promotion of rapid absorption of drugs and nutrients. These included increased blood supply to the gastrointestinal tract or increased levels of enzymes such as γ-glutamyl transpeptidase (γGT), which participate in active and passive transport of nutrients to the intestinal cells (Jori et al, 1992)

Pharmacokinetics of piperine

The pharmacokinetics of piperine in humans remains incompletely understood. In rat piperine is absorbed following ingestion, and some metabolites have been identified, piperonylic acid, piperonyl alcohol, piperonal and vanillic acid are found in the urine. One metabolite, piperic acid, is found in the bile (Sharma et al, 2005).

CHAPTER III

MATERIALS AND METHODS

Materials

1. Chemical

Bovine insulin [Sigma]

Dimethyl sulfoxide (DMSO) [BDH]

5-5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) [Sigma]

Dulbecco's Modified Eagle's Medium (DMEM) [Sigma]

Fetal Bovine Serum [Hyclone]

Glutathione reductase [Sigma]

Glutathione reduced form [Sigma]

Hoechst 33342 [Sigma]

Hydrogen peroxide [Sigma]

In vitro toxicology assay kit [Sigma]

Lactate dehydrogenase (LDH) Kit [Sigma]

MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) [Sigma]

β-Nicotinamide adenine dinucleotide phosphate, reduced form [β-NADPH] [Sigma]

Na_2SeO_3 [Sigma]

Penicillin G [Sigma]

Piperine [Sigma]

Poly-D-Lysine Hydrobromide [Sigma]

Progesterone [Sigma]

Propidium iodide [Sigma]

Putrescine [Sigma]

Sulfosalicylic acid [Sigma]

Streptomycin [Sigma]

0.4% Trypan Blue [Sigma]

Sodium phosphate buffer [Sigma]

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

TBA reagent [sigma]

Transferrin [Sigma]

2. Instruments

Adjustable pipette : 10-100 μl [Nichiyo]

Adjustable pipette : 200-1000 μl, 1-5 ,ml [Labsystems]

Aluminum Foil [Tops]

Bottles, glass 100 ml, 500 ml, 1000 ml [Schott Duran]

Bunsen burner

Carbon dioxide incubator [Forma Scientific]

Cell culture dish: diameter 35 mm, diameter 100 mm [Nunc]

24-well cell culture plate [Nunc]

Centrifuge

Conical tube: 15 ml, 50 ml [Nunc]

Disposable glass Pasteur pipette : 230 mm [Volac]

Hemocytometer (Depth 0.100 mm) [Improved Neubauer]

Inverted microscope, Axiovent [Zeiss]

Latex Free Syringe: 10 ml [Becton Dickinson]

Laminar air flow hood [Hepaco]

96-well microtiter plate [Nunc]

Microliter pipette : 10-100 μl, 200-1000 μl, 1-5 ml [Labsystems]

Microplate reader [Biorad model 3550]

pH meter

Pipettes tip : 1-200 μl, 200-1000 μl, 1-5 ml [Labsystems]

Sonicator [Elma]

Spectrophotometer

Sterile Millex-GV (0.22 μm filter unit) [Millipore]

Sterivex-GS (0.22 μm filter unit with filling bell) [Millipore]

Syringe filter holder : 13 mm [Satorius]

Water distiller

Water bath

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Experimental Methods

1. Preparation of culture media, buffers and solutions

1.1 Culture media

Dulbecco's modified Eagle's medium (DMEM, high glucose) was used in N1E-115 cell line culture. DMEM was supplemented with 3.7 mg/ml sodium bicarbonate, 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. Fetal bovine serum (FBS) was added into Serum-free DMEM to a final concentration of 10% (v/v). Serum–free DMEM composed of normal DMEM without phenol red, supplemented with 30 μg/ml transferrin, 5 μg/ml insulin, 100 μM putrescine, 20 nM progesterone and 30 nM sodium selenite (N-2 supplement). The media was adjusted to pH 7.3 using 1 N NaOH and 1 N HCl.

1.2 Phosphate buffered saline solution (PBS)

For 1 liter preparation, it consisted of KCl 0.20 g, KH_2PO_4 0.2 g, NaCl 8 g, Na_2HPO_4 1.150 g, and EDTA 0.37 g. The solution was adjusted to pH 7.4 using 1 N NaOH and 1 N HCl.

1.3 0.1 M sodium phosphate buffer

The 0.1 M Sodium phosphate buffer was used for dissolving 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and NADPH. It contained 94.7 ml of 0.2 M sodium phosphate dibasic and 5.3 ml of 0.2 M sodium phosphate monobasic. The solution volume was adjusted to 200 ml by distilled water and adjusted to pH 7.5 using 1 N NaOH and 1 N HCl.

1.4 Hydrogen peroxide solution

30% H_2O_2 solution (equivalent to 9.794 M) was freshly diluted with PBS to desired concentrations before adding to cultured neuronal cell line.

1.5 Piperine solution

In all experiments, piperine was dissolved in DMSO for treatment of cultured cells. The final concentration of DMSO in culture medium was $\leq$ 0.5%. All solutions were sterilized through a 0.22 μm Millipore filter. Control cultures were treated with equivalent amounts of DMSO.

2. N1E-115 Cell line Culture

Murine neuroblastoma cells, clone N1E-115 (passage numbers 23-30), were cultured in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% (v/v) fetal bovine serum and incubated in an atmosphere of 5%`CO_2/95% humidified air at 37 ^{o}C. When the cultures reached confluency (approximately 2-3 days), cells were removed from the flasks by trypsinization. The culture medium was aspirated and cells were further incubated with 0.05% trypsin-EDTA solution for 2 min with gentle agitation, followed by the addition of an equal volume of DMEM containing 10% FBS. The cell suspension was centrifuged at 3,000 rpm for 5 min and the pellet was suspended in fresh DMEM containing 10% FBS. The cells were dissociated by passing through a flame-polished Pasteur pipette and viable cells were counted on a hemocytometer using trypan blue exclusion. After diluting with culture medium, aliquots of 200 μl were plated at a density of 2.5 x 10^3 cells/well in 96-well culture plates and cultures were incubated in an atmosphere of 5% CO_2/95% humidified air at 37 ^{o}C. After growth to confluency [approximately 2-3 days after plating], the cells were used in experiments.

3. Exposure of N1E-115 cell line cultures with hydrogen peroxide

Cultures were exposed to H_2O_2 at final concentrations of 50, 100, 200, 300, 400, 500, and 1000 μM in serum-free, N2-supplemented DMEM for 1, 3, 6, 12, and 24 hr. Thereafter, mitochondrial metabolic activity was assayed by MTT reduction and cell death was assayed by lactate dehydrogenase (LDH) release.

4. Treatment of N1E-115 cell culture with piperine

Cultures were treated with piperine at concentrations of 0.1-20 μM for 1, 3, 6, 12, or 24 hr in serum-free, DMEM containing N2 supplements. Thereafter, mitochondrial metabolic activity was assayed by MTT reduction and cell death was assayed by lactate dehydrogenase (LDH) release.

5. Effects of piperine on H_2O_2-induced cell injury and death

5.1. Pre-treatment with piperine

Cultures were initially incubated with nontoxic concentrations of piperine in serum-free DMEM containing N2 media supplements, for 12 or 24 hr. Then, the culture medium was replaced with fresh serum-free, N2-supplemented DMEM containing an insulting concentration of H_2O_2 for a predetermined duration.

5.2. Co-treatment with piperine

Cultures were incubated with an insulting concentration of H_2O_2 for a predetermined duration in combination with nontoxic concentrations of piperine in serum-free DMEM containing N2 media supplement.

6. Effects of piperine on H_2O_2-induce lipid peroxidation

Optimum protective concentration of piperine was selected for further study on H_2O_2-induce lipid peroxidation in cultured N1E-115 cell line cultures. Thiobarbituric acid reactive substances (TBARS) assay was used to determine levels of cellular lipid peroxidation.

7. Effects of piperine on H_2O_2-induced glutathione depletion

Optimum protective concentration of piperine was selected for further study on H_2O_2-induced glutathione depletion in cultured N1E-115 cell line cultures using total glutathione assay.

8. Effects of piperine on H_2O_2-induced apoptotic cell death

Optimum protective concentration of piperine was selected for further study on H_2O_2-induced apoptotic cell death in cultured N1E-115 cell line cultures using a two color fluorescence DNA staining.

9. Analytical Methods

9.1. MTT reduction assay

Cellular dehydrogenase activity which is considered to reflect mitochondrial activity was measured by the reduction of tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide to formazan. Mitochondrial dehydrogenase in viable cells converts the soluble yellow form of the salt into an insoluble, intracellular purple formazan which is quantitated spectrophotometrically by solubilization in an organic solvent.

MTT reduction was analyzed by adding 16 μl of the MTT stock solution [5 mg/ml in phosphate-buffered saline (PBS)] to the medium in each well (final concentration was 100 μg/ml). Cultures were incubated in a CO_2 incubator at 37 ^{o}C for 1 hr and the medium in each well was aspirated off without disturbing the formazan precipitate. Then 400 μl of DMSO was added to each well in order to solubilize the formazan crystals. Following thorough formazan solubilization, 200 μl aliquots of soluble formazan were transferred to a 96-well microtiter plate. The cellular reduction of MTT was determined by measuring the absorbance at 595/655 nm with a BIO-RAD Model 3550 dual wavelength microplate reader. The results were expressed as percentage of MTT reduction relative to that of control culture.

9.2. Lactate dehydrogenase release assay

Released LDH is a biologically stable enzymatic marker that correlates linearly with cell death. Cell viability was determined by assaying the medium from each well for lactate dehydrogenase (LDH) activity using an *in vitro* toxicology assay kit according to the manufacture's instructions. The assay is based on the reduction of NAD by the LDH-catalyzed conversion of lactate to pyruvate. Then reduced NAD (NADH) is then utilized in the conversion of a tetrazolium dye. The resulting colored compound is measured spectrophotometrically.

Briefly, medium LDH was assayed by pipetting 100 μl of culture medium from each well into a 96-well microtiter plate. Cellular LDH was measured by carefully aspirated off the remaining culture medium and solubilized cells with 1 ml of 0.5% Triton X-100 in PBS after which 100 μl aliquots were pipetted into a 96-well microtiter plate. The reaction was started by adding 50 μl of lactate dehydrogenase assay mixture into each well. The plate was covered with an aluminum foil to protect from light and incubated at room temperature for 30 min. The reaction was terminated by the addition 50 μl of 1N HCl into each well. The light absorbance in each well was measured at 490/655 nm with a BIO-RAD Model 3550 dual wavelength microplate reader. The total activity of LDH in control cultures was considered to represent 100% cell viability. The LDH release which reflects cell death was presented as percentage of total LDH activity by the following formula:

$$\%\ \text{LDH release} = \frac{\text{LDH activity in medium} \times 100}{\text{LDH activity in medium} + \text{LDH activity in cells}}$$

In most cases, comparative LDH release in test conditions was expressed as the percentage of that in control conditions.

9.3. Glutathione assay

DTNB

2GSH

glutathione reductase

GSSG

2 5-mercapto-2-nitrobenzoic acid

λmax:412 nm

Cellular GSH levels were measured enzymatically by using a modification of the procedure of Tietze (1969). This method is based on the determination of a chromophoric product, 2-nitro-5-thiobenzoic acid, resulting from the reaction of 5-5'-dithiobis-(2-nitrobenzoic acid) with GSH. In this reaction, GSH is oxidized to GSSG, which is then reconverted to GSH in the presence of GSH reductase (GRx) and NADPH. The rate of 2-nitro-5-thiobenzoic acid formation is followed at 450 nm (Tietze, 1969; Baker et al, 1990). Cells were washed with ice-cold PBS and immediately collected by scraping with 0.3 ml of 1% (wt/vol) sulfosalicylic acid. Cell lysates were transferred to 1.5-ml Eppendorf tubes and centrifuged at 12,000 rpm for 5 min at 4 ^{o}C. Following cell extraction, 10 μl aliquots of supernatant were transferred into 96-well microtiter plate and the volume was made up to 100 μl with 0.1 M phosphate buffer containing 1 mM EDTA (pH 7.5). The reaction was started by adding 100 μl of reaction mixture [0.3 mM 5,5′-dithio-bis (2-nitrobenzoic acid), 0.4 mM NADPH, and 1 U/ml glutathione reductase in 0.1 M sodium phosphate buffer (pH 7.5)]. The formation of 2-nitro-5-benzoic was monitored at 30-s intervals for 10 min at 450/620 nm by using a microplate reader. The slope of the initial rate of reaction was used for calculating GSH content from a standard curve obtained by plotting known amount of GSH (25-150 μM). The cellular content of GSSG was typically less than 2% of the GSH level and was not considered.

9.4. Thiobarbituric acid reactive substances (TBARS) assay

The sensitivity of measuring thiobarbituric acid reactive substances (TBARS) has made this assay the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress. Malondialdehyde (MDA), an end product of polyunsaturated fatty acid oxygenation, is a reliable and commonly used biomarker for assessing lipid peroxidation. MDA forms a 1:2 adduct with thiobarbituric acid (TBA) and produces a colored MDA-TBA adduct which can be measured by fluorometry or spectrophotometry.

TBARS was measured using a technique modified from Ohkawa et al. (1979) and Storch et al. (2002). Briefly, cultured cells were washed three times with Hank's balanced salt solution (HBSS) and lysed with 160 μl of 2% sodium dodecyl sulfate for 30 min.Cell lysates from six cell culture wells were pooled, transferred to glass tube and adjusted with 2% sodium dodecyl sulfate to 1 ml. The lysates were added serially with 50 μl of butylated hydroxytoluene (4% in ethanol), 1 ml of phosphotungstic acid (10% in 0.5 M sulfuric acid) and 1.5 ml of thiobarbituric acid (0.7%). The mixtures were incubated at 95° C for 60 min, cooled by tap water, extracted with 4 ml of n-butanol and centrifuged at 3,500 rpm for 10 min. After centrifugation, the fluorescence of the n-butanol layer was measured at 515 nm excitation and 553 nm emission with a spectrofluorometer. Results are expressed in term of percentage control.

9.5. apoptotic cell death assay

N1E-115 cell line was cultured in 96-well plates (2.5 x 10^3 / well). Cultures were detected for apoptotic cells by a two-color fluorescence staining with two nuclear fluorochromes, the membrane-permeant bisbenzimide dye Hoechst 33342 (Ho 33342) which labels nuclei of all cells, and the membrane-impermeant dye propidium iodide (PI) which labels only nuclei of dead cells. By this method it was possible to detect, in the same sample and at the same time, intact cells, cells undergoing apoptosis, and dead cells resulting from apoptotic and/or necrotic processes.

Cells were stained according to the one-step staining procedure. The staining involving several dye combinations have been applied to a variety of cell types. By controlling the dye concentrations it is possible to minimize the dye-dye interactions as well as the excessive background fluorescence. Ho 33342 (1 mg/ml stock solution in distilled water) was added to the cultures to give a final concentration of 1 μg/ml. Simultaneously, PI (500 μg/ml stock solution in distilled water) was also added to the cultures, to give a final concentration of 5 μg/ml. The cultures were incubated in a CO_2 incubator for 15 min at 37 °C. After removing the medium the neurons were rinsed once with phosphate buffer solution (PBS, pH 7.2) and incubated in cooled PBS. The stained cells were immediately analyzed under UV fluorescence optics with an inverted microscope and the photographs were obtained. In each culture, six to ten consecutive microscopic fields (200×) were counted. To determine the proportion of dead or apoptotic neurons, at least 200 cells were counted per culture. Numbers of cells in different stages were presented as the percentage of total cell count. Whenever possible, comparisons were made on matched sister cultures derived from single plating. The criteria for identification of cells in different states are as described in the table.

Table 1. Criteria for the simultaneous assessment of apoptotic and necrotic cells by the two-color fluorescence DNA staining.

Cell subpopulation	Ho 33342 fluorescence (blue)	PI fluorescence (red)
Viable	Intermediate	Negative
Early apoptotic or	Increased, condensed or fragmented	Negative
Late apoptotic or	Decreased, condensed or fragmented	Positive, condensed or fragmented
Dead or Necrotic or	Much decreased	Highly positive

10. Statistical analysis

All results were presented as the means ± SEM values from 6 to 8 independent cultures, with duplicate replications in each experiment. Differences among means were analyzed using one-way analysis of variance (ANOVA). One-way ANOVA followed by Sheffe test was used for planned multiple comparisons. All statistical analysis was performed according to the instruction for the statistical program SPSS. A value of $P < 0.05$ was considered to be significant.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER IV

RESULTS

1. Cytotoxic effects of H_2O_2 on N1E-115 cell line cultures

To assess the cytotoxic potential of H_2O_2, N1E-115 cell line cultures were exposed to H_2O_2 for 1-24 hr at 50-1000 μM H_2O_2 induce changes in mitrochondrial metabolic activity (MTT reduction assay) and cell death (LDH release assay). It was apparent that exposure to H_2O_2 at 50-1000 μM for 3-24 hr showed cytotoxic effects in a concentration- and exposure time dependent manner (Figures 5 and 6).

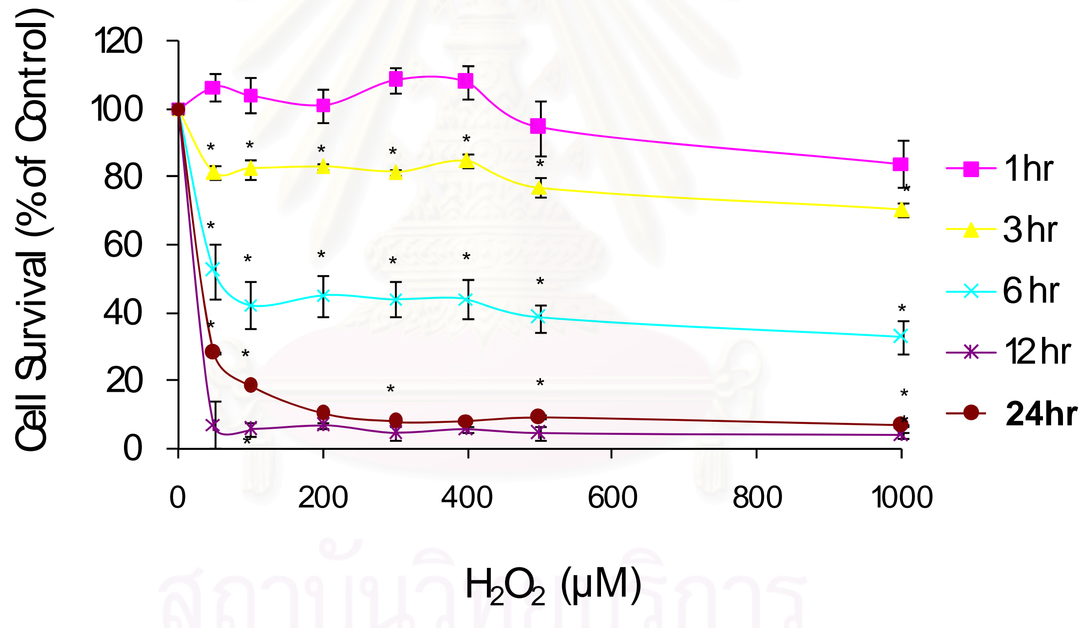

Figure 5. Effects of H_2O_2 on cell survival in N1E-115 cell line cultures.

Cultures were exposed to H_2O_2 (50-1000 μM) for indicated times. Cell viability was assessed by MTT reduction assay. Data are expressed as mean ± SEM of six independent experiments carried out in duplicate. Statistical comparison was made using one-way ANOVA followed by Sheffe test for multiple comparison. *$p < 0.05$ vs control cultures.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

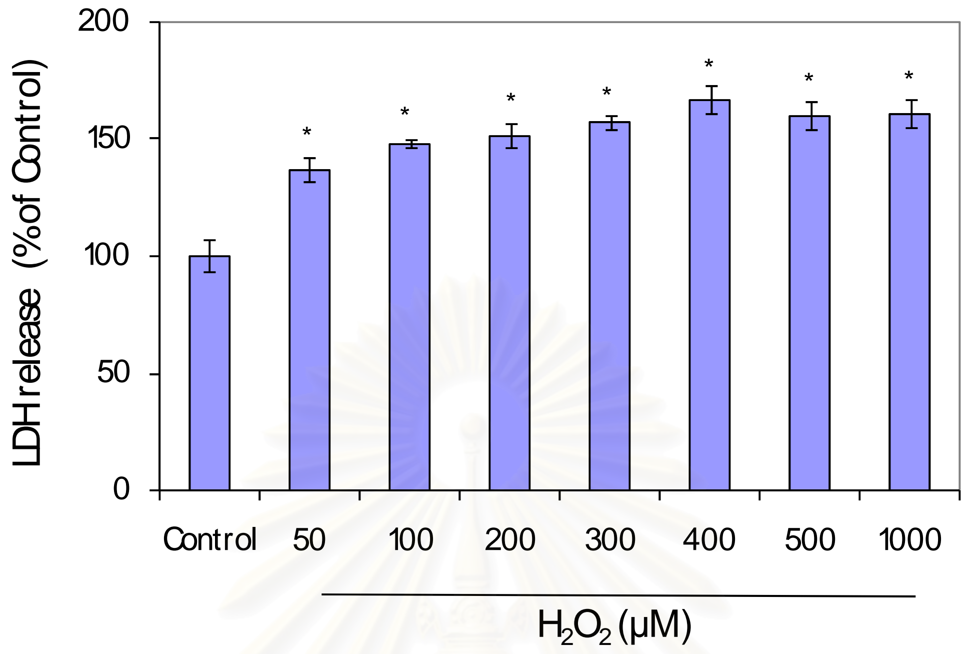

Figure 6. Effect of H_2O_2 on cell death in N1E-115 cell line cultures.

Cultures were exposed to H_2O_2 (50-1000 mM) for 6 hr. Cell death was assessed by LDH release assay. Data are expressed as mean ± SEM of six independent experiments carried out in duplicate. Statistical comparison was made using one-way ANOVA followed by Sheffe test for multiple comparison. *$p < 0.05$ vs control cultures.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

2. Effects of piperine on N1E-115 cell line cultures

Exposure of N1E-115 cell line cultures to piperine (0.1-20 μM) for 1-24 hr induced modest changes in mitochondrial metabolic activity (MTT reduction assay) and cell death (LDH release assay). At concentrations of 0.1-10 μM, piperine did not induce any significant changes in cell metabolic activity or cell death during 1-24 hr exposure. After exposure to piperine at 15-20 μM for 12-24 hr, cultured neurons showed only significantly decreased mitochondrial metabolic activity without changes in cell death (Figures 7 and 8).

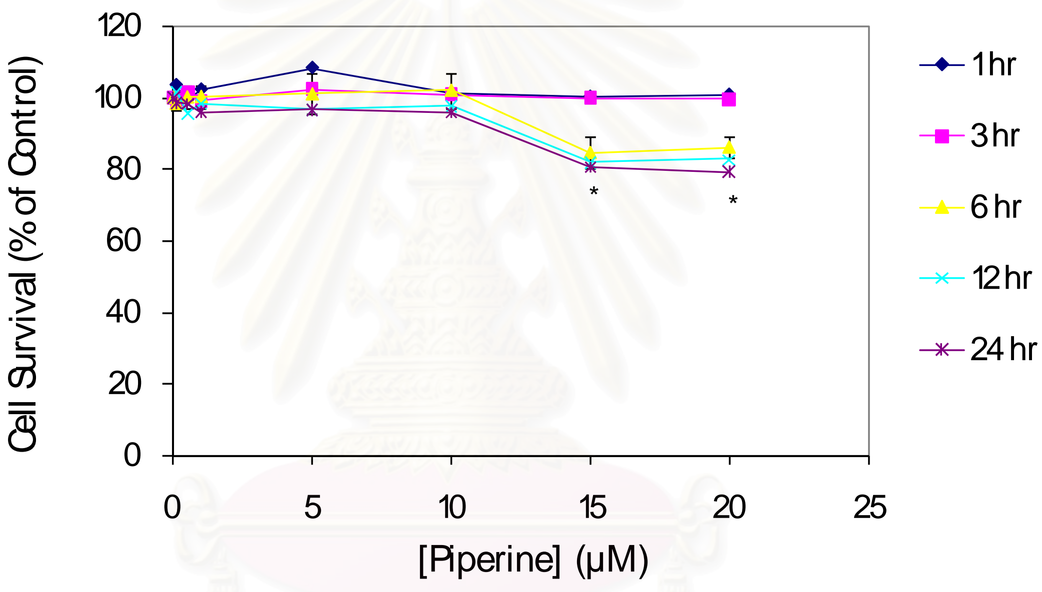

Figure 7. Effects of piperine on cell survival in N1E-115 cell line cultures.

Cultures were exposed to piperine (0.1-20 μM) for indicated times. Cell viability was assessed by MTT reduction assay. Data are expressed as mean ± SEM of six independent experiments carried out in duplicate. Statistical comparison was made using one-way ANOVA followed by Sheffe test for multiple comparison. *$p < 0.05$ vs control cultures.

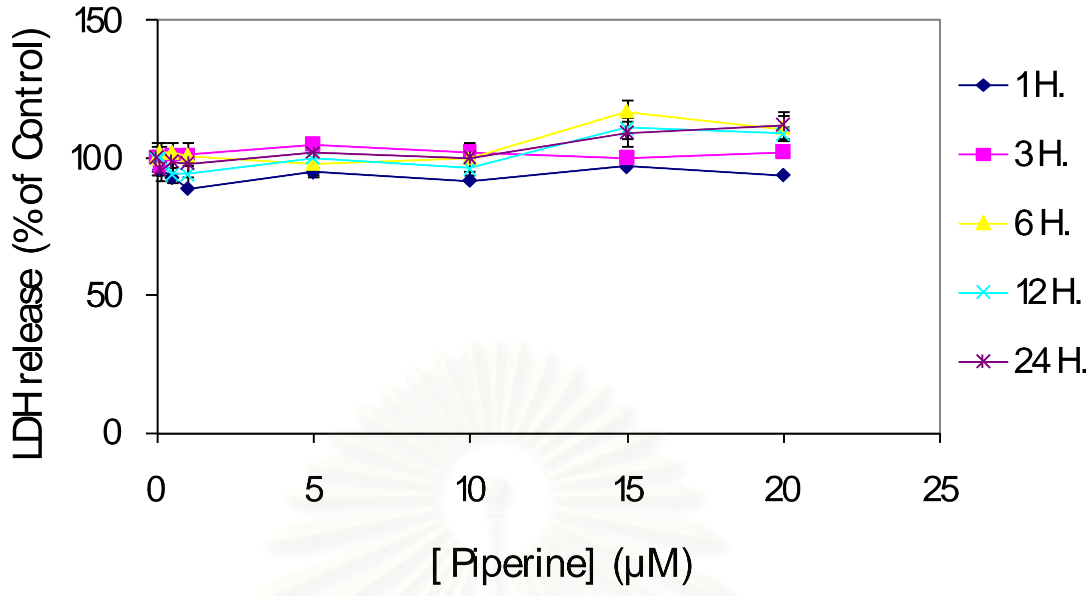

Figure 8. Effects of piperine on cell death in N1E-115 cell line cultures.

Cultures were exposed to piperine (0.1-20 µM) for indicated times. Cell death was assessed by LDH release assay. Data are expressed as mean ± SEM of six independent experiments carried out in duplicate. Statistical comparison was made using one-way ANOVA followed by Sheffe test for multiple comparison.

3. Effect of pre-exposure with piperine on H_2O_2-induced cell injury and death in N1E-115 cell line cultures

Pre-exposure of cultured neurons with 0.1-10 μM piperine (nontoxic concentrations) for 12 or 24 hr before submission to H_2O_2 exposure at 50 μM for 6 hr (insulting conditions) did not show any beneficial effects on cell metabolic activity (Figure 9) or cell death (Figure 10) as compared to H_2O_2-treated group without pretreatment.

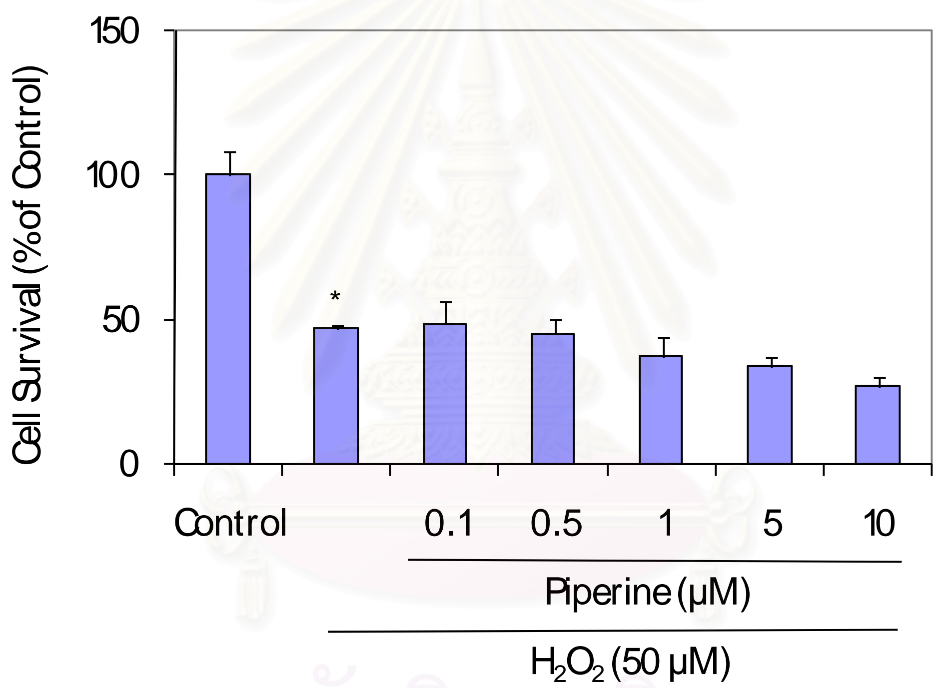

Figure 9. Effect of 12 hr pre-exposure with piperine on H_2O_2-induced cell injury in N1E–115 cell line cultures.

Cultures were incubated with piperine for 12 hr before switching to 50 μM H_2O_2 for 6 hr. Cell survival was assessed by MTT assay. The data was expressed as percentage of control value. Statistical comparison was made using one-way ANOVA followed by Sheffe test for multiple comparison. Six independent experiments were carried out in duplicate. *P < 0.05 vs control group.

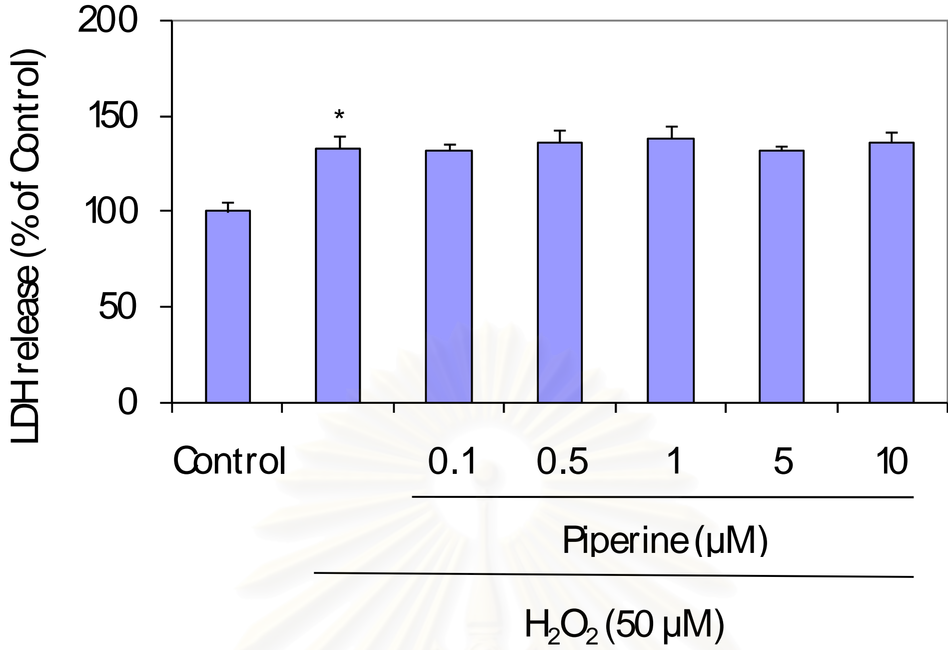

Figure 10. Effect of 12 hr pre-exposure with piperine on H_2O_2-induced cell death in N1E–115 cell line cultures.

Cultures were incubated with piperine for 12 hr before switching to 50 μM H_2O_2 for 6 hr. Cell viability was assessed by LDH release assay. The data was expressed as percentage of control value. Statistical comparison was made using one-way ANOVA followed by Sheffe test for multiple comparison. Six independent experiments were carried out in duplicate. *P < 0.05 vs control group.

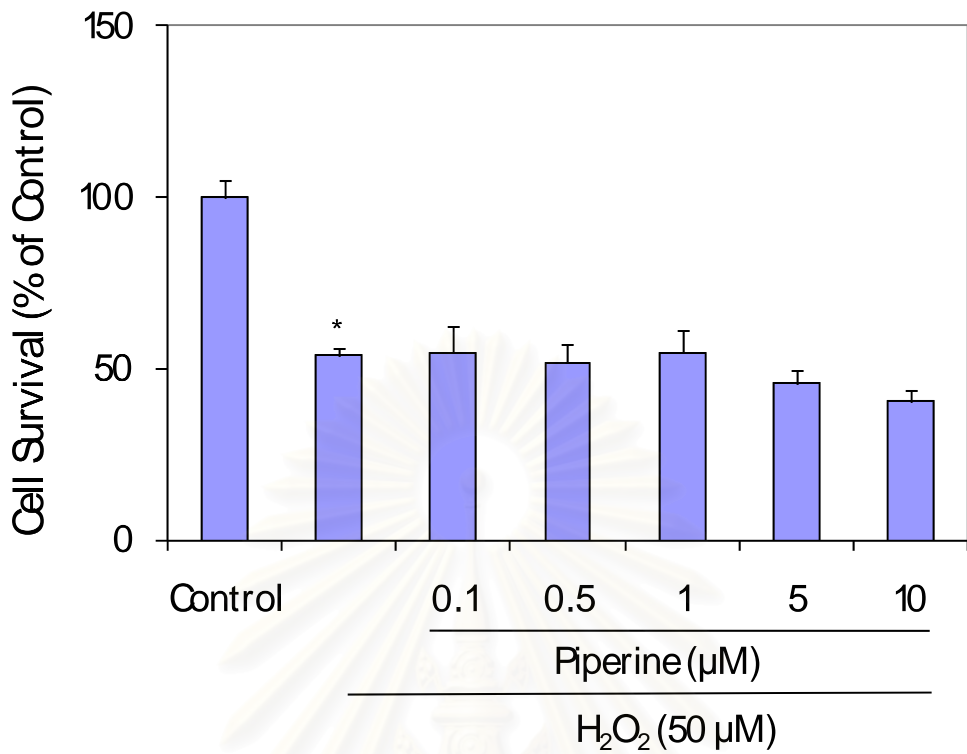

Figure 11. Effect of 24 hr pre-exposure with piperine on H_2O_2-induced cell injury in N1E–115 cell line cultures.

Cultures were incubated with piperine for 24 hr before switching to 50 μM H_2O_2 for 6 hr. Cell survival was assessed by MTT assay. The data was expressed as percentage of control value. Statistical comparison was made using one-way ANOVA followed by Sheffe test for multiple comparison. Six independent experiments were carried out in duplicate. *$P < 0.05$ vs control group.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

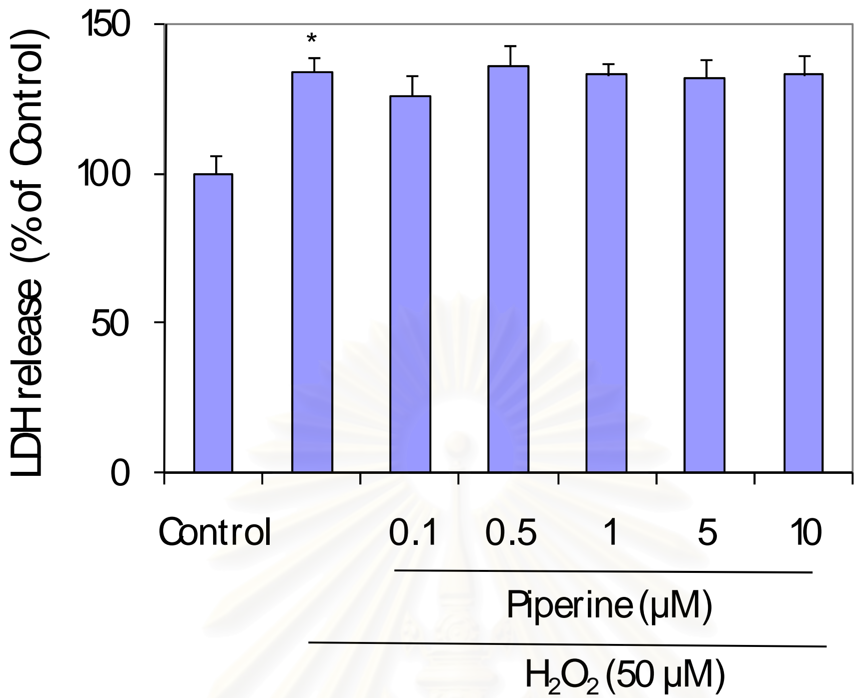

Figure 12. Effect of 24 hr pre-exposure with piperine on H_2O_2-induced cell death in N1E–115 cell line cultures.

Cultures were incubated with piperine for 24 hr before switching to 50 μM H_2O_2 for 6 hr. Cell viability was assessed by LDH release assay. The data was expressed as percentage of control value. Statistical comparison was made using one-way ANOVA followed by Sheffe test for multiple comparison. Six independent experiments were carried out in duplicate. *P < 0.05 vs control group.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

4. Effect of co-exposure with piperine on H_2O_2-induced cell injury and death in N1E-115 cell line cultures

Co-exposure of cultured neurons with 0.1-10 μM piperine (nontoxic concentrations) with 50 μM H_2O_2 for 6 hr (insulting conditions) revealed beneficial effects on cell metabolic activity (Figure 13) or cell death (Figure 14) as compared to H_2O_2-treated group without pretreatment. Neuroprotective effect of piperine was markedly apparent at piperine concentrations of 5-10 μM.

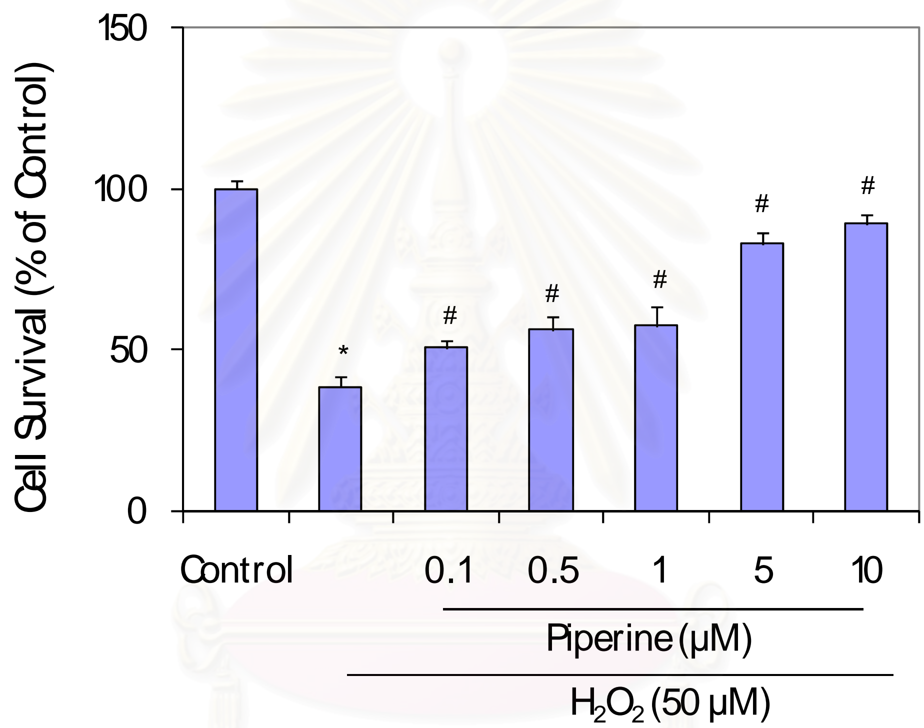

Figure 13. Effect of co-exposure with piperine on H_2O_2-induced cell injury in N1E–115 cell line cultures.

Cultures were simultaneously incubated with piperine and 50 μM H_2O_2 for 6 hr. Cell survival was assessed by MTT assay. The data was expressed as percentage of control value. Statistical comparison was made using one-way ANOVA followed by Sheffe test for multiple comparison. Six independent experiments were carried out in duplicate. *$P < 0.05$ vs control group; $^{\#}P < 0.05$ vs H_2O_2 group.

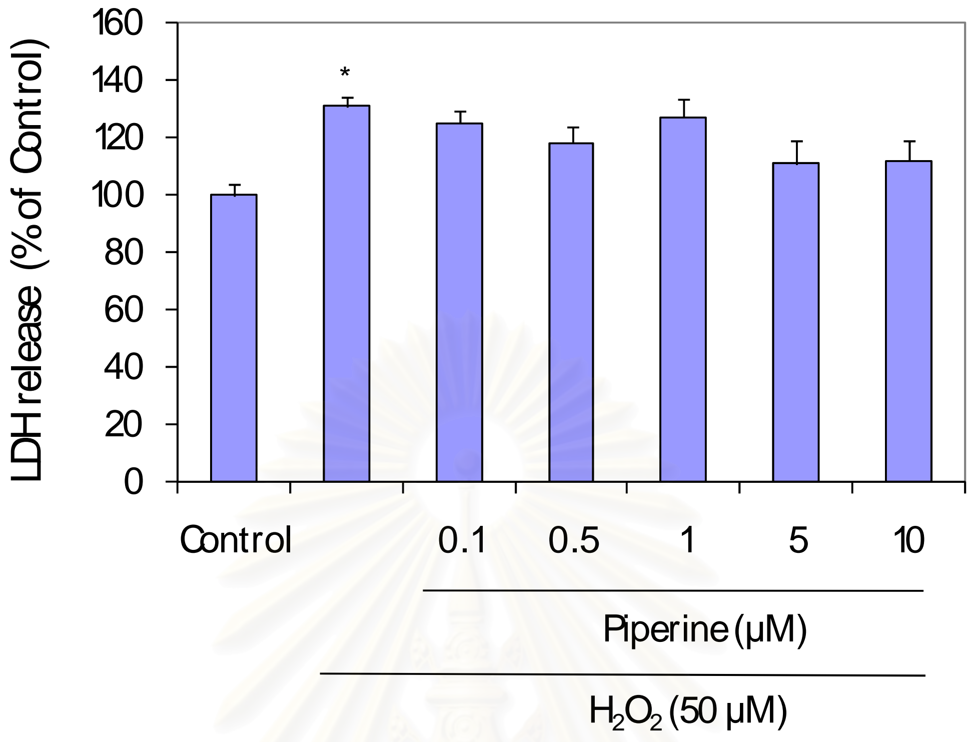

Figure 14. Effect of co-exposure with piperine on H_2O_2-induced cell death in N1E–115 cell line cultures.

Cultures were simultaneously incubated with piperine and 50 μM H_2O_2 for 6 hr. Cell death was assessed by LDH release assay. The data was expressed as percentage of control value. Statistical comparison was made using one-way ANOVA followed by Sheffe test for multiple comparison. Six independent experiments were carried out in duplicate. *P < 0.05 vs control group.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

5. **Effects of co-exposure with piperine on H_2O_2-induced lipid peroxidation in N1E-115 cell line cultures**

Treatment of N1E-115 cell line cultures with 50 μM H_2O_2 for 6 hr increased cellular levels of lipid peroxidation. Intracellular MDA, a product of lipid peroxidation, was markedly increased to approximately 185% of control cultures. However, after coexposure with 10 μM piperine, levels of Intracellular MDA were significantly decreased as compared with H_2O_2-treated group (Figure 15).

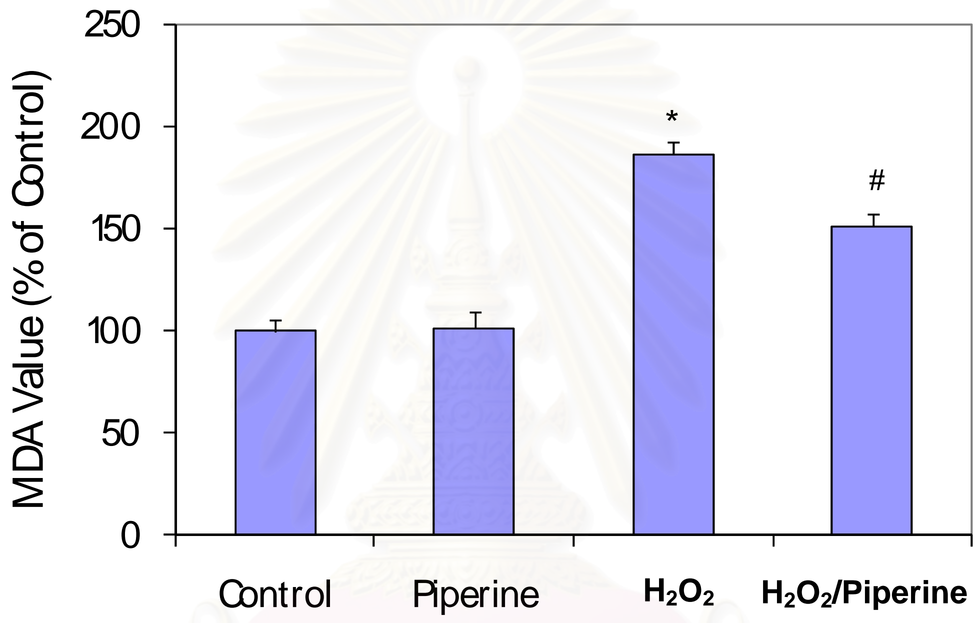

Figure 15. Effect of co-exposure with 10 μM piperine on 50 μM H_2O_2-induced lipid peroxidation in N1E-115 cell line cultures. Values are expressed as percentage of control value. Statistical comparison was made using one-way ANOVA followed by Sheffe test for multiple comparison. Six independent experiments were carried out in duplicate. *P < 0.05 vs control group; #P < 0.05 vs H_2O_2 group.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

6. Effect of co-exposure with piperine on H_2O_2-induced depletion of GSH in N1E-115 cell line cultures

Treatment of N1E-115 cell line cultures with 50 μM H_2O_2 for 6 hr decreased cellular contents of GSH to approximately 57% of control cultures. After coexposure with 10 μM piperine, cellular contents of GSH MDA were modestly increased as compared to H_2O_2-treated group. However, this increase was not statistically significant (Figure 16).

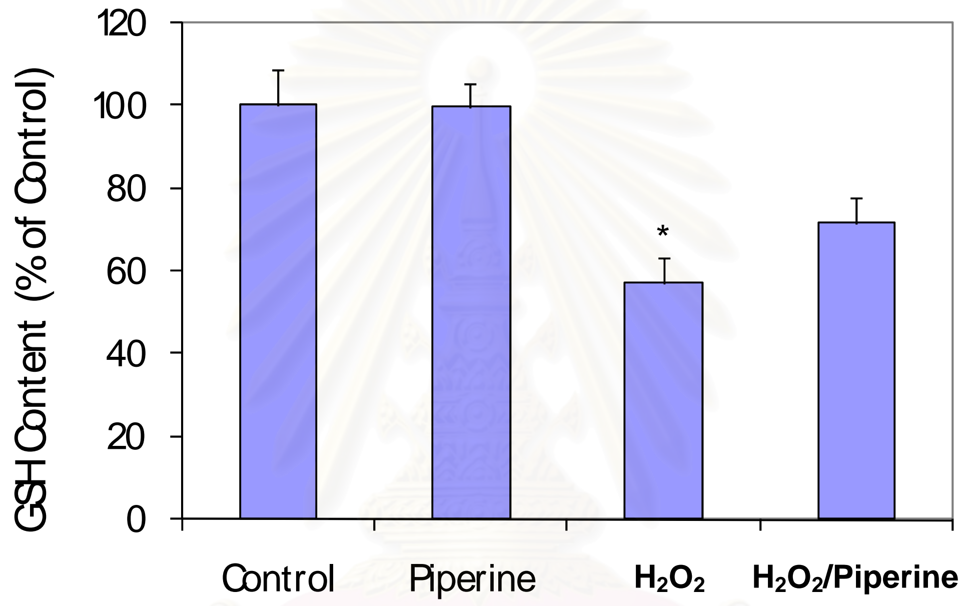

Figure 16. Effect of co-exposure with 10 μM piperine on 50 μM H_2O_2-induced depletion of GSH in N1E-115 cell line cultures. Values are expressed as percentage of control value. Statistical comparison was made using one-way ANOVA followed by Sheffe test for multiple comparison. Six independent experiments were carried out in duplicate. $^*P < 0.05$ vs control group.

6. Effect of co-exposure with piperine on H_2O_2-induced apoptotic cell death in N1E-115 cell line cultures

Treatment of N1E-115 cell line cultures with 50 μM H_2O_2 for 6 hr increased number of apoptotic cells as compared to control cultures. After coexposure with 10 μM piperine, number of apoptotic cells was modestly decreased as compared to H_2O_2-treated group. However, this decrease was not statistically significant (Figure 17).

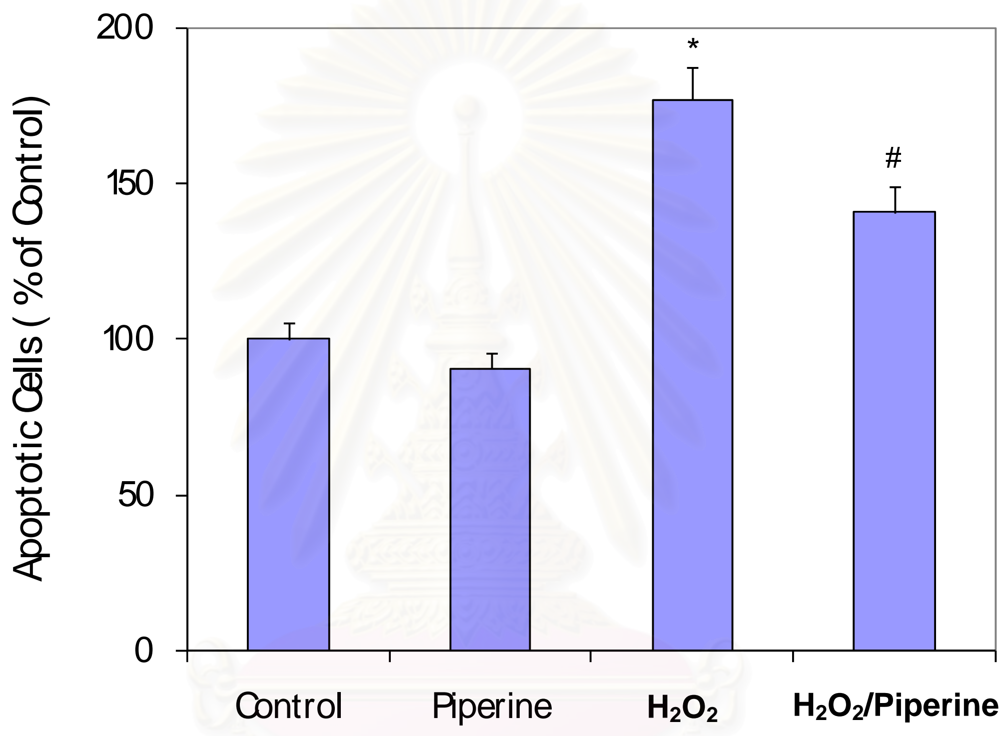

Figure 17. Effect of co-exposure with 10 μM piperine on 50 μM H_2O_2-induced apoptotic cell death in N1E-115 cell line cultures. Values are expressed as percentage of control value. Statistical comparison was made using one-way ANOVA followed by Sheffe test for multiple comparison. Six independent experiments were carried out in duplicate. *$P < 0.05$ vs control group; #$P < 0.05$ vs H_2O_2 group.

CHAPTER V

DISCUSSION AND CONCLUSION

Oxidative stress-induced cell damage has been implicated in a variety of neurodegenerative disorders such as stroke, Alzheimer's disease, and Parkinson's disease. It is mediated by ROS, including H_2O_2, superoxide and hydroxyl radicals, which are generated as byproducts of normal and aberrant metabolic processes that utilize molecular oxygen. ROS can attack proteins, deoxynucleic acid, and lipid membranes, thereby disrupting cellular function and integrity. It has been demonstrated that ROS cause cell death via apoptosis. There are many types of chemical and physiological inducers of oxidative stress which are able to cause apoptotic death. For instance, H_2O_2 can induce apoptosis in many different cell types (Whittemore et al., 1994; Whittemore et al., 1995), and this effect can be blocked by addition of antioxidants (Jang and Surh, 2001). As the major component of ROS, H_2O_2 has been extensively used as an inducer of oxidative stress in the *in vitro* models (Satoh et al., 1996). The delineation of biochemical pathways involved in neuronal cell death due to H_2O_2 may aid in the development of drugs for treatment of various neurodegenerative diseases.

A cellular model of neurodegeneration induced by H_2O_2 was used to investigate the oxidative stress-induced neuronal cell death in this study. It had been shown that exposure to various concentrations (50-1000 μM) of H_2O_2 for different time intervals (1-24 hr) caused marked cell injury and death in N1E-115 cell line cultures in a concentration- and exposure time-dependent manner. An appropriate condition of H_2O_2–induced neurotoxicity was selected for further study on the potential neuroprotective effects of piperine. An exposure to 50 μM H_2O_2 for 6 hr was considered to be optimal because it decreased mitochondrial metabolic activity to approximately 50% of the control cultures and significantly increased number of cell death. It was notable that after exposure to various concentrations of H_2O_2 for 12-24 hr, metabolic activity of neuronal mitochondria was severely suppressed. Therefore, H_2O_2 is a strong insulting agent and requires careful monitoring of culture condition for effective use as a cellular model of neurodegeneration. Cytotoxicity of H_2O_2 had been shown to mediate

through different mechanisms. H_2O_2 is one of products of reactive oxygen species (ROS) which can cause oxidative stress in mammals. This oxidative stress has been examined as regards its association with the pathology of neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease. H_2O_2 has also been related to several pathological conditions such as Huntington's disease, human immunodeficiency virus infection and apoptosis. H_2O_2 has also been shown to modify neurotransmitter release at synaptic terminals. Therefore, it is conceivable to use H_2O_2 as an insulting agent to understand the process and mechanism of neuronal damages. In particular, H_2O_2 may be selectively more toxic to immature neurons (Mischel et al, 1997).

Although neuronal cultures are simplified models of neurodegenerative disorders (e.g., cerebral hypoxia-ischemia, cerebrovascular stroke), they enable investigation of specific processes involved in the complex, multifactorial pathogenesis of these injuries. Furthermore, neurons developing *in vivo* and *in vitro* share important characteristics including developmentally regulated vulnerability to neurotoxicity and maturation-dependent expression of antioxidant enzymes. The similarities between neurons developing *in vivo* and *in vitro* suggest that studies of H_2O_2-induced neuronal injury in culture may provide insights into the unique pathogenesis of perinatal hypoxic-ischemic brain injury. Definition of the specific mediators of perinatal hypoxic-ischemic brain injury may ultimately enable the development of effective clinical therapies in the management of this common and often devastating condition.

Piperine has been reported to possess various pharmacological activities, such as modulation of carcinogen-induced oxidative stress in intestinal mucosa (Khajuria et al, 1998). In this study, preliminary results revealed that exposure to 0.1-10 μM piperine for 1-24 hr had no cytotoxic effect on N1E-115 cell line cultures as considered from MTT reduction and LDH release assays. Whereas exposure to 15-20 μM piperine exhibited cytotoxic effect by decreasing mitochondrial metabolic activity and increasing number of cells death. Thus piperine seems to have bimodal effects on neuronal cells depending mainly on its concentrations. Piperine may exert cytoprotective effects at relatively low concentrations whereas it appears to be cytotoxic at higher concentrations (Unchern et al, 1994a, 1994b, 1997, 1998; Wonganan, 2002; Pensirinapa, 2002). The ambiguous

questions so far are what is the borderline between the “good” and “bad” properties of piperine and how does it exert both types of effects. In addition, is it possible to attain cytotoxic levels of piperine by daily consumption of pepper in specific groups of population? Accumulated evidence suggests that *in vitro* exposure of cells to 0.1-10 μM of piperine for less than 6 hr is relatively nontoxic. Moreover, vulnerability to cytotoxic effects of piperine may depend on cell types and maturity.

Co-exposure of nontoxic concentrations of piperine with toxic concentrations of H_2O_2 was apparently beneficial to cultured neurons. Under this particular situation, piperine attenuated H_2O_2-induced suppression of mitochondrial metabolic activity and apoptotic cell death in conjunction with a marked reduction in cellular lipid peroxidation which was generated by exposure to H_2O_2. However, pre-exposure to piperine for 12-24 hr before switching to H_2O_2 exposure did not show any beneficial effects to cultured neurons. This finding suggests that pretreatment with piperine may be unable to induce the expression of antioxidant enzymes or proteins in immature neurons or neurons *in vitro*. Thus, apparent neuroprotective effects of piperine may involve its antioxidant and scavenging properties which is in accordance with its *in vivo* effects on memory deficits and lipid peroxidation in an animal model of cerebral ischemia (Pensirinapa, 2002). Piperine at doses of 0.1 and 0.5 mg/kg/day, injected intraperitoneally for 5 consecutive days, was effective in preventing brain lipid peroxidation increase and deficits in spatial memory in mice with temporary bilateral carotid artery occlusion while similar dosage regimens with 1 and 5 mg/kg/day had no effects. It was apparent that piperine was more effective at preventing memory deficit at lower doses than higher doses. This particular dose-response characteristic which is apparent both in the *in vitro* and *in vivo* experiments may be due to the bimodal nature of piperine (antioxidant vs pro-oxidant) depending on its concentrations at the site of action.

In contrary, pre-exposure to 2.5 - 10 μM piperine for 24 hr protected cultured glial cells from LPS-induced inhibition of mitochondrial metabolic activity (Wonganan, 2002). This discrepancy may be due to a difference in cell type and maturity. Cultured glial cells are mature and well-equipped with antioxidant systems while cultured N1E-115 cells are immature and deficient in effective antioxidant mechanisms.

Inhibition of lipid peroxidation by piperine had been previously reported. Piperine significantly reduced the membrane and plasma lipid peroxides in cancer bearing animals (Selvendriran and Sakthisekaran, 2004). There is extensive evidence that supplementation of piperine can enhance antioxidant enzymes and other seleno proteins (Dhuley et al, 1993). The present *in vitro* study supports a possible short-term action of piperine in addition to long-term actions that previously predicted. Piperine might act as a hydroxyl radical and superoxide scavenger (Rascher, et al, 2000) or, alternatively, interfere with Fenton reaction thereby decreasing generation of ROS.

The tripeptide γ-glutamylcysteinylglycine or GSH is the major nonenzymatic regulator of intracellular redox homeostasis, ubiquitously present in all cell types at millimolar concentration (Meister and Anderson, 1983). This cysteine-containing tripeptide exists either in reduced (GSH) or oxidized (GSSG) form, better referred to as glutathione disulfide, and participates in redox reactions by the reversible oxidation of its active thiol. Under normal cellular redox conditions, the major portion of this regulator is in its reduced form and is distributed in nucleus, endoplasmic reticulum and mitochondria. In addition, GSH may be covalently bound to proteins through a process called glutathionylation and acts as a coenzyme of numerous enzymes involved in cell defense. Glutathione can thus directly scavenge free radicals or act as a substrate for glutathione peroxidase (GPx) and glutathione S-transferase (GST) during the detoxification of hydrogen peroxide, lipid hydroperoxides and electrophilic compounds.

GSH is abundant in neurons and acts as a major cellular antioxidant. Reduction of the intracellular GSH content enhances oxidative stress and eventually results in cell death. Results from many studies showed that H_2O_2 causes GSH depletion and neuronal death. In this study, exposure of N1E-115 cells to H_2O_2 resulted in GSH diminution in similar manner to that previously reported. If 10 μM piperine was simultaneously present with H_2O_2, GSH diminution was partially counteracted but this difference was not statistically significant. Piperine might be able to activate the cellular antioxidative system by modulating the expression of some antioxidant enzymes. However, this activity can differ among different cells or pathological conditions. In this particular case, N1E-115 cell line cultures may be inappropriate for a study on GSH metabolism.

Apoptosis is a form of cell death that plays important roles during normal mammalian development and tissue homeostasis. Apoptotic cell death is characterized typically by morphological alterations, including chromatin condensation and apoptotic body formation, and DNA fragmentation. Apoptotic cell death is composed of several processes, including an activation phase and a degradation phase. It has been proposed that oxidative stress mediates a final common pathway of apoptotic cell death.

The most identifiable biochemical event in apoptosis is the nuclear condensation and internucleosomal fragmentation of DNA through an endonuclease which results in the formation of DNA fragments. The presence of chromatin condensation and fragmentation as determined by the two-color fluorescence DNA staining, provide a convincing evidence for H_2O_2-induced apoptosis.

A simple and rapid fluorescence method modified from that used in the flow cytometry of apoptotic cells was employed for quantitating the number of apoptotic and necrotic neurons. In cell culture, unlike *in vivo*, apoptotic cells are not phagocytosed and eventually lyse (postapoptotic or secondary necrosis). This is of practical importance because morphological changes seen in cultures may be due to the transition from apoptosis to necrosis, rather than to the induction of apoptosis itself. With the two-color fluorescence DNA staining, distinguishing between different stages of typical necrosis and apoptosis is generally easy and accurate on the basis of morphological features. Necrotic cells are PI-positive (red), showing generally swollen and round nuclei without any nuclear condensation. Early apoptotic cells are not stained by PI but exhibit classical nuclear alterations (condensation and fragmentation) visualized by Ho 33342 stain (blue). However, during late stage of apoptosis, neurons progressively lost their cell membrane integrity and become PI-positive. These neurons show red-orange apoptotic nuclei due to transition of fluorescence color from blue to red (PI fluorescence plus quenching of the Ho 33342 fluorescence by energy transfer to PI). In late stage of necrosis, cells are progressively disintegrated into small fragments and, similarly, late apoptotic cells or apoptotic bodies undergo postapoptotic necrosis. These small and indiscernible fragments with red-orange fluorescence were not counted, therefore, the analysis mainly considers relatively early steps of necrosis and is probably only slightly

distorted by postapoptotic necrosis. Conversely, necrotic cells probably did not interfere in the count of apoptotic cells. Therefore, although it is likely that postapoptotic necrosis occurs during the time of the experiments, the possible interference of postapoptotic necrosis in the analysis is probably minor.

H_2O_2-induced cell death via apoptosis had been reported by several studies (Kim et al, 2006; Jiang et al, 2003; Uberti et al, 1999; Whittemore et al, 1995). Reactive oxygen species (ROS) are continuously generated *in vivo* but increases in their steady states are regarded to be responsible for a variety of pathological conditions, including cardiovascular disease, cancer, and aging. Among a great variety of ROS, H_2O_2 plays a pivotal role because it is generated from nearly all sources of oxidative stress and can diffuse freely in and out of cells and tissues. Moreover, it has been shown that H_2O_2 has the ability to modulate signal transduction pathways, to change the homeostasis of ions such as calcium and iron, to activate several transcriptional factors with consequent expression of a great number of genes, to provoke cell proliferation and differentiation, and finally to induce cell death either by apoptosis or necrosis.

Cellular DNA is especially sensitive to the action of H_2O_2 and this DNA damage is widely believed to be mediated by transition metal ions, mainly iron and/or copper, which are able to catalyze the formation of hydroxyl radicals ($HO^{\bullet}$) by Fenton-type reactions. The location of redox-active metals is likely to be of utmost importance for the ultimate effect because HO•, due to their extreme reactivity, interact exclusively in the vicinity of the bound metal. Formation of HO• close to DNA (due to bound Fe or Cu ions) results in its damage, including base modifications, single- and double- strand breakage, and sister chromatid exchange. However, there are indications that location of iron at positions other than DNA may contribute indirectly to DNA damage and ensuing apoptosis. Extensive work has shown that lysosomal iron may be a key player in peroxide-dependent cell damage and apoptosis. Moreover, results from our laboratory as well as others, indicated that the formation of single-strand breaks in cellular DNA was Ca^{2+}-dependent, indicating an obligatory intermediary role for Ca^{2+} and, consequently, a signaling pathway leading to DNA damage.

It is generally believed that extensive DNA damage leads invariably to cell death either by apoptosis or necrosis. In particular, H_2O_2 may induce both apoptosis and necrosis depending on the concentration of the oxidant employed and/or the type of the cell being studied. It has to be noted, however, that in the vast majority of studies with H_2O_2 it was added directly to the cells as a bolus, so that cells were initially exposed to relatively high concentrations followed by a fast decrease as H_2O_2 is gradually consumed. Consequently, if the mode of action of H_2O_2 is concentration dependent (as most probably is the case), the results might appear inconsistent.

In this study, the cellular insulting condition may be relatively mild. Exposure to 50 μM H_2O_2 for 6 hr markedly increased the extent of apoptotic cell death in N1E-115 cell line cultures. This increase was significantly attenuated by co-exposure with 10 μM piperine. Therefore, overall evidence suggests that piperine may possess neuroprotective effects against H_2O_2-induced cell injury and apoptotic cell death. On one hand, piperine may counteract at some points in apoptotic cascade. On the other hand, a decrease in apoptotic cell death may be a consequence of reduced cellular oxidative stress caused by piperine.

CONCLUSION

Experimental results from this study suggest that simultaneous exposure with lower concentrations of piperine, in a range of 0.1-10 μM, may be protective against cell injury and apoptotic cell death from exposure to H_2O_2 in N1E-115 cell line cultures. The mechanism of action underlying this beneficial effect of piperine on cultured neurons is unclear at the present time. Possibly, it may involve different direct and indirect actions of piperine, e.g., antioxidation and free radical scavenging, interference with Fenton's reaction which generates ROS, inhibition of cellular lipid peroxidation, counteraction to apoptotic cell death cascade.

REFERENCES

Ahmad S. (ed.) 1995. Oxidative stress and antioxidant defenses in biology. Chapman & Hall, New York.

Atal C.K., Dubey R.K. and Singh J. 1985. Biochemical basis of enhanced drug bioavailability by piperine: evidence that piperine is a potent inhibitor of drug metabolism. J. Pharmacol. Exp. Ther. 232:258-262.

Badmaev V., Majeed M., and Norkus EP. 1999. Piperine, an alkaloid derived from black pepper increase serum response of beta-carotene during 14-days of oral beta-carotene supplementation. Nutr. Res. 19:381-388.

Badmaev V., Majeed M., and Prakash L. 2000. Piperine derived from black pepper increases the plasma levels of Coenzyme Q_{10} following oral supplementation. J. Nutr. Biochem. 11:109-113.

Baker M. A., Cerniglia G. J. and Zaman A. 1990. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Anal. Biochem 190: 360-365.

Bano G., Raina R. K., Zutshi U., Bedi K.L., Johri R.K., and Sharma S.C. 1991. Effect of piperine on bioavailability and pharmacokinetics of propranolol and theophylline in healthy volunteers. Eur. J. Clin. Pharmacol. 41:615-617.

Behl C. and Moosmann B. 2000. Antioxidant neuroprotection in Alzheimer's disease as preventive and therapeutic approach. Free Radical Biol.Med. 33:182-191

Bothwell M. and Giniger E. 2000. Alzheimer's disease: Neurodevelopment converge with neurodegeneration. Cell 102:271-273.

Brown R.C., Han Z.C., and Papadopolos M. 2003. Oxidative stress mediated DHEA formation in Alzheimer's disease pathology. Neurobiol. Aging 24:57-65.

Butterfield D.A., Griffin S., Munch G., Pasinetti. G.M. 2002. Amyloid beta peptide and amyloid pathology are central to the oxidative stress and inflammatory cascades under which Alzheimer's disease brain exists. J. Alzheimer's Dis. 4:193-201

Chen Y.C., Chow J.M., Lin C.W., Wu C.Y., and Shen S.C. 2006. Baicalein inhibition of oxidative-stress-induced apoptosis via modulation of ERKs activation and induction of HO-1 gene expression in rat glioma cells C6. Toxicol. Appl. Pharmacol. 216:263-273.

David M. 2003. Singlet oxygen-mediated damage to proteins and its consequences. Biochemical and Biophysical research communications. 305:761-770.

DeRosa M.C. and Crutchley R.J. 2002. Photosensitized singlet oxygen and its applications. Coord. Chem. Rev. 233-234:351-371.

D'Hooge R., Pei Y. Q., Raes A., Lebrun P., van Bogaert P.P., and de Deyn P.P. 1996. Anticonvulsant activity of piperine on seizures induced by excitatory amino acid receptor agonists. Arzneim. Forsch. 46:557-560.

Dhuley J.N., Raman P.H., Mujumdar A.M., and Naik S.R. 1993. Inhibition of lipid peroxidation by piperine during experimental inflammation in rat. Indian J. Exp. Biol. 31:443-445.

Drake J., Link C.D., and Butterfield D.A. 2003. Oxidative stress precedes fibrilla deposition of Alzheimer's disease amyloid beta-peptide (1-42) in a transgenic *Caenorrhabditis elegans* models. Neurobiol. Aging. 24:415-420.

Fujita Y., Izawa Y., Ali N., Kanematsu Y., Tsuchiya K., Hamano S., Tamaki T., Yoshizumi M. 2006. Pramipexole protects against H_2O_2-induced PC12 cell. Naunyn Schmiedebergs Arch. Pharmacol. 372: 257-266.

Govindarajan V.S. 1977. Pepper chemistry, technology and quality evaluation. Crit. Rev. Food Sci. Nutr. 9:115-225.

Bijur G.N., Davis R.E., and Jope R.S. 1999. Rapid activation of heat shock factor-1 DNA binding by H_2O_2 and modulation by glutathione in human neuroblastoma and Alzheimer' s disease cybrid cells. Mol. Brain Res. 71: 69-77.

Halliwell B. 1992. Reactive oxygen species and the central nervous system. J. Neurochem. 59:1609-1632.

Hinshaw D.B., Miller M. T., Beals T.F., and Hyslop P.A. 1993. A cellular model of oxidant-mediated neuronal injury. Brain Res. 615:13-26.

Jang J.H., and Surh Y.J. 2001. Protective effects of resveratrol on hydrogen peroxide-induced apoptosis in rat pheochromocytoma (PC12) cells. Mutation Res. 496:181–190.

Jiang B., Liu J.H., Bao Y.M., and An L.J. 2003. Hydrogen peroxide-induced apoptosis in pc12 cells and the protective effect of puerarin. Cell Biol. Int. 27:1025–1031.

Jochen R., Stohr., Pei-Gen Xiao., and Bauer R. 2001. Constituents of Chinese *Piper* species and their inhibitory activity on prostaglandin and leukotriene biosynthesis *in vitro*. J. Ethnopharmacol. 75:133-139.

Joe B. and Lokesh B.R. 1994. Role of capsaicin, curcumin and dietary n-3 fatty acids in lowering the generation of reactive oxygen species in rat peritoneal macrophages. Biochim. Biophys. Acta 1224:255-263.

Johri R.K., Thusu N., Khajuria A. and zutshi U. 1992. Piperine-mediated changes in the permeability of rat intestinal epithelial cells, the status of γ-glutamyl transpeptidase activity, uptake of amino acids and lipid peroxidation. Biochem. Pharmacol. 43:1401-1407.

Khajuria A., Thusu N., Zutshi U., and Bedi K.L. 1998. Piperine modulation of carcinogen induced oxidative stress in intestinal mucosa. Mol. Cell Biochem. 189:113-118.

Kim D.S., Jeon S.E., Jeong Y.M., Kim S.Y., Kwon S.B., and Park K.C. 2006. Hydrogen peroxide is a mediator of indole-3-acetic acid/horseradish peroxidase-induced apoptosis. FEBS Lett. 580:1439-1446.

Krantic S., Mechawar N., Reix S., and Quirion R. 2007. Apoptosis-inducing factor: A matter of neuron life and death. Prog. Neurobiol. 81:179–196.

Krishnakantha T.P. and Lokesh B.R. 1993. Scarvenging of superoxide anions by spice principles. Indian J. Biochem. Biophys. 30:133-134.

Koul I.B. and Kapil A. 1992. Evaluation of the liver protective potential of piperine, an active principle of black and long peppers. Planta Med. 59:413-417.

Kulshrestha V.K., Singh N., Srivastava R.K., Rastogi S.K., and Kohli R.P. 1971. Analysis of central stimulant activity of *Piper longum*. J. Res. Ind. Med. 6:17-23.

Lennon S.V., Martin S.J., and Cotter T.G. 1991. Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. Cell. Prolif. 24: 203-214.

Landsberg R.C. and Vollgraff U. 1998. Mode of cell injury and death after hydrogen peroxide exposure in cultured oligodendroglia cells. Exp. Cell Res. 244:218-229.

Lee E.B., Shin K.H. and Woo W.S. 1984. Pharmacological study on piperine. Arch. Pharmacol. Res. 7:127-132.

Liu G.Q., Algeri S., Ceci A., Garattini S., Gobbi M., and Murai S. 1984. Stimulation of serotonin synthesis in rat brain after antiepilepsirine, an antiepileptic piperine derivative. Biochem. Pharmacol. 33:3883-3886.

Lossi L. and Merighi A. 2003. *In vivo* cellular and molecular mechanisms of neuronal apoptosis in the mammalian CNS. Prog. Neurobiol. 69:287–312.

Malini T., Manimaran R.R., Arunakaran J., Aruldhas M.M., and Govindarajulu P. 1999. Effects of piperine on testis of albino rats. J Ethnopharmacol. 64:219-225.

Roberfroid M. and Calderon P.B. (eds.) 1995. Free radicals and oxidation phenomena in biological systems. Mercel Dekker Inc, New York.

Martenson M.E.., Arguells J.H., and Baumann T.K. 1997. Enhancement of rat trigeminal ganglion neuron responses to piperine in a low-pH environment and block by capsazepine. Brain Res. 761:71-76.

McBride A.G., Borutaite V., and Brown G.C.1999. Superoxide dismutase and hydrogen peroxide cause rapid nitric oxide breakdown, peroxynitrite production and subsequent cell death. Biochim. Biophys. Acta. 1454:275-288.

Meister A, and Anderson M.E. 1983. Glutathione. Annu. Rev. Biochem. 52:711–760.

Mischel R.E., Kim Y.S., Sheldon R.A., and Ferriero D.M. 1997. Hydrogen peroxide is selectively toxic to immature murine neurons *in vitro*. Neuroscience Lett. 231:17–20.

Mittal R. and Gupta R. L. 2000. *In vitro* antioxidant activity of piperine. Meth. Find. Exp. Clin. Pharmacol. 22:271-274.

Mori A., Kabuto H., and Pei Y. Q. 1985. Effect of piperine on convulsions and on brain serotonin and catecholamine levels in E1 mice. Neurochem. Res. 10:1269-1275.

Mujumdar A.M., Dhuley J.N., Desmukh K., Raman P.H., and Naik S.R. 1990a. Anti-inflammatory activity of piperine. Jpn. J. Med. Sci. Biol. 43:95-100.

Mujumdar A.M., Dhuley J.N., Desmukh K., Raman P.H. and Naik S.R. 1990b. Effect of piperine on pentobarbitone induced hypnosis in rats. Indian J. Exp. Biol. 28:486-487.

Ohkawa H., Ohishi N., and Yagi, K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 98: 351-358.

Olanow C.W. 1992. An introduction to the free radical hypothesis in Parkinson's disease. Ann. Neurol. S2-9.

Olanow C.W. 1993. A radical hypothesis for neurodegeneration. Trends Neurosci. 16:439-444.

Pei Y.Q. 1979. A study of central pharmacological action of N-(*p*-chlorocinnamoyl)-piperidine and N-(cinnamoyl)-piperine. J. Beijing Med. College 4:234-238.

Pei Y.Q. 1983. A review of pharmacology and clinical use of piperine and its derivatives. Epilepsia 24:177-182.

Pei Y. Q., Yue W., Cui J. R., and Yao H.Y. 1980. Study on the central pharmacological effect of piperine and its derivatives. Yao Hsueh Hsue Pao 15:198-205.

Pelsman A., Hoyo-Vadillo C., Gudasheva T.A., Seredenin S.B.., Ostrovskaya R.U., and Busciglio J. 2003. GVS-111 prevents oxidative damage and apoptosis in normal and Down's syndrome human cortical neurons. Int. J. Devl Neuroscience. 21:117-124.

Pensirinapa S. 2002. Effect of piperine on learning and memory impairment induced by transient cerebral ischemia or scopolamine in mice. Master Thesis ISBN 974-17-2958-8.

Piyachaturawat P., Glinsukon T., and Toskulkao C. 1983. Acute and subacute toxicity of piperine in mice, rats and hamsters. Toxicol. Lett. 16:351-359.

Piyachaturawat P., Kingkaeohoi S., and Toskulkao C. 1995. Potentiation of carbon tetrachloride hepatotoxicity by piperine. Drug Chem. Toxicol. 18:333-344.

Pradeep C.R. and Kuttan. 2004. Piperine is a potent inhibitor of nuclear factor-κB (NF-κB), c-Fos, CREB, ATF-2 and proinflammatory cytokine gene expression in B16F-10 melanoma cells. Int. Immunopharmacol. 4:1795-1803.

Rauscher F.M., Sander R.A., and Watkins J.B. 2000. Effects of piperine on antioxidant pathways in tissue from normal and streptozotocin-induced diabetic rats. J. Biochem. Mol. 14:329-334.

Ratan R.R., Murphy T.H., and Baraban J.M. 1994. Oxidative stress induces apoptosis in embryonic cortical neurons. J. Neurochem. 62:376-379.

Reddy A.C. and Lokesh B.R. 1992. Studies on spice principles as antioxidants in the inhibition of lipid peroxidation of rat liver microsomes. Mol. Cell Biochem. 111:117-124.

Richardson S.J. 1993. Free radicals in the generation of Alzheimer's disease. Ann N.Y. Acad. Sci. 695:73-76.

Satoh I., Sakai N., Enokido Y., Uchiyama Y., and Hatanaka H., 1996. Free radical independent protection by nerve growth factor and Bcl-2 of PC12 cells from hydrogen peroxide triggered-apoptosis. J. Biochem. 120:540–546.

Schreck R. and Bauerle P.A. 1991. A role for oxygen radicals as second messengers. Trends Cell Biol. 1: 39-42.

Selvendiran K., Banu S., and Sakthisekaran D. 2004. Protective effect of piperine on benzo (a) pyrene-induced lung carcinogenesis in Swiss albino mice. Clinica Chimica Acta. 350:73-78.

Selvendiran K. and Sakthisekaran D. 2004. Chemopreventive effect of piperine on modulating lipid peroxidation and membranes in benzo (a) pyrene-induced lung carcinogenesis. Biomed. Pharmacother. 58:264-267.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Sharma P., Varma M. V., Chawla H. P., and Panchagnula R. 2005. *In situ* and *in vivo* efficacy of peroral absorption enhancers in rats and correlation to *in vitro* mechanistic studies. Farmaco 60:874-883.

Shenoy N.R. and Choughuley A.S. 1992. Characterization of potentially mutagenic products from the nitrosation of piperine. Cancer Lett. 64:235-239.

Shoba G., Joy D., Joseph T., Majeed M., Rajendran R., and Srinivas P.S. 1998. Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. Planta Med. 64:353-356.

Singh N., Kulshrestha V.K., Srivastana R.K. and Kohli R.P. 1973. Studies on the analeptic activity of some *Piper longum* alkaloids. J. Res. Ind. Med. 8:1-9.

Storch A., Burkhardt K., Ludolpg A.C., and Schwarz J. 2002. Protective effects of riluzole on dopamine neurons: involvement of oxidative stress and cellular energy metabolism. J. Neurochem. 75: 2259-5569.

Sunila E.S. and Kuttan G. 2004. Immunomodulatory and antitumor activity of *Piper longum* Linn. and piperine. J. Ethnopharmacol. 90:339-346.

Teepker M., Anthes N., Fisher S., Krieg J.C., and Vedder H. 2007. Effects of oxidative challenge and calcium on ATP-levels in neuronal cells. Neurotoxicology 28:19-26.

Tietze F. 1969. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal. Biochem. 27: 502-522.

Uberti D., Yavin E., Gil S., Ayasola K-R., Goldfinger N., and Rotter V. 1999. Hydrogen peroxide induces nuclear translocation of p53 and apoptosis in cells of oligodendroglia origin. Mol. Brain Res. 65:167–175.

Unchern S., Nagata K., Saito H. and Fukuda, J. 1994a. Piperine, a pungent alkaloid, is cytotoxic to cultured neurons from the embryonic rat brain. Biol. Pharm. Bull 403-406.

Unchern S., Nagata K., Saito H., and Fukuda J., 1994b. Reduction of neurite growth by piperine, examined on hippocampal and septal neurons of the embryonic rat brain. Biol. Pharm. Bull. 17:898-901.

Unchern S., Saito H., and Nishiyama N. 1997. Selective cytotoxicity of piperine on cultured rat hippocampal neurons in comparison to cultured astrocytes: The possible involvement of lipid peroxidation. Biol. Pharm. Bull. 20:958-961.

Unchern S., Saito H., and Nishiyama N. 1998. Death of cerebellar granule neurons induced by piperine is distinct from that induced by low potassium medium. Neurochem. Res. 23:97-102

Valencia A., Moran J. 2004. Reactive oxygen species induce different cell death mechanism in cultured neurons. Free Rad. Biol. Med. 36:1112-1125.

Vijayakumar R.S., Surya D., and Nalini N. 2004. Antioxidant efficacy of black pepper (*Piper nigrum* L.) and piperine in rats with high fat diet induced oxidative stress. Redox Rep. 9:105-110.

Whittemore E.R., Loo D.T., and Cotmans C.W., 1994. Exposure to hydrogen peroxide induces cell death via apoptosis in cortical culture. NeuroReport. 5:1485-1488.

Whittemore E.R., Loo D.T., Watt J.A., Cotmans C.W. 1995. A detailed analysis of hydrogen peroxide-induced cell death in primary neuronal culture. Neuroscience 67:921-932.

Wonganan P. 2002. Effect of piperine on lipopolysaccharide-induced injuries and oxidative changes in rat brain glial cell cultures. Master Thesis ISBN 974-17-3019-5

Wright A., HawKins C., and David M. 2003. Photo-oxidation of cells generates long-life intracellular protein peroxides. Free Rad. Biol. Med. 34:637-647.

Wrona M., Korytowski W., Rozanowska M., Sarna T., and Truscott M. 2003. Cooperation of antioxidants in protection against photosensitized oxidation. Free Rad. Biol. Med. 35:1319-1329.

Woo W.S., Lee E.B., and Shin K.H. 1979. Central nervous system depressant activity of piperine. Arch. Pharmacol. Res. 2:121-125.

Xie J. and Guo Q. 2004. AATF protects neural cells against oxidative damage induced by amyloid β-peptide. Neurobiol. Dis. 16:150-157.

Yamada M., Momose K., Richelson E., and Yamada M. 1996. Sodium nitroprusside-induced apoptotic cellular death via production of hydrogen peroxide in murine neuroblastoma N1E-115 cells. J. Pharmacol. Toxicol. Meth. 35:11-17.

Zhou J.Y., Liu Y., and Wu G.S. 2006. The role of mitogen-activated protein kinase phosphatase-1 in oxidative damage-induced cell death. Cancer Res. 66:4888-4894.

Zhu T., Chen R., Li A., Liu J., Gu D., Liu Q., Chang H.C., and Zhou J. 2006. JWA as a novel molecule involved in oxidative stress-associated signal pathway in myelogenous leukemia cells. J. Toxicol. Environ. Health A. 69:1399-1411.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDICES

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table1 Effect of H_2O_2 on MTT reduction in cultured N1E-115 cells

Table1.1 1 hr of incubation

%viability	%control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
PBS	106	97	97	104	79	95	96	4
50	106	99	108	122	110	93	106	4
100	98	91	101	127	105	102	104	5
200	98	88	121	107	110	83	101	5
300	102	89	117	118	114	111	109	4
400	88	97	118	121	108	117	108	5
500	72	62	115	112	105	102	95	8
1000	65	57	102	102	87	91	84	7

N = number of experiments (duplicated)

Table1.2 3 hr of incubation

%viability	%Control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
PBS	92	86	90	102	100	96	95	2
50	81	82	88	83	77	78	81	2
100	97	82	81	83	75	75	82	3
200	87	87	78	83	80	82	83	1
300	78	83	79	87	81	80	81	1
400	98	81	83	83	83	80	85	2
500	85	69	70	87	77	75	77	3
1000	65	78	68	67	74	67	70	2

N = number of experiments (duplicated)

Table1.3 6 hr of incubation

%viability	% control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
PBS	99	104	99	88	98	101	98	2
50	81	77	39	40	37	40	52	8
100	65	69	30	31	29	29	42	7
200	75	50	28	43	40	34	45	6
300	53	63	34	44	39	31	44	5
400	51	63	30	40	41	29	44	6
500	52	49	26	38	35	29	38	4
1000	56	42	21	34	26	18	33	5

N = number of experiments (duplicated)

Table1.4 12 hr of incubation

%viability	% Control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
PBS	91	87	92	95	116	104	98	4
50	6	7	5	7	9	7	7	0.4
100	5	6	4	6	6	6	5	0.3
200	7	7	6	6	7	7	7	0.3
300	4	5	3	5	3	5	4	0.4
400	6	7	5	5	6	6	6	0.2
500	4	7	2	4	4	5	4	0.6
1000	5	5	2	4	4	5	4	0.4

N = number of experiments (duplicated)

Table1.5 24 hr of incubation

%viability	% Control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
PBS	95	84	81	98	89	96	91	3
50	15	21	29	10	59	36	28	7
100	15	19	14	9	27	24	18	2
200	12	7	11	10	12	7	10	1
300	15	6	9	8	5	3	8	2
400	14	5	9	8	8	5	8	1
500	16	11	9	8	7	4	9	2
1000	12	3	6	8	6	6	7	1

N = number of experiments (duplicated)

Table2 Effect of H_2O_2 on LDH release in cultured N1E-115 cells for 6 hr

%viability	% Control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
PBS	95	110	88	103	113	103	101	3
50	124	149	127	137	156	135	137	5
100	124	156	156	151	176	132	148	2
200	153	139	141	148	178	148	151	5
300	154	161	144	158	158	171	157	3
400	140	149	155	151	183	169	167	6
500	160	165	136	164	182	163	160	6
1000	154	185	145	157	171	163	161	5

N = number of experiments (duplicated)

Table3 Effect of piperine on MTT reduction in cultured N1E-115 cells

Table3.1 1 hr of incubation

%viability	% Control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
DMSO	99	98	104	106	97	105	101	1
0.1	105	106	99	116	101	92	103	3
0.5	109	105	102	86	97	96	99	3
1	107	102	103	124	91	85	102	6
5	116	104	102	128	96	102	108	5
10	106	95	90	117	108	90	101	4
15	103	95	97	113	96	97	100	3
20	100	98	98	113	97	98	101	2

N = number of experiments (duplicated)

Table3.2 3 hr of incubation

%viability	% control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
DMSO	107	101	116	91	85	100	100	5
0.1	99	95	122	87	87	105	99	5
0.5	120	111	92	88	101	97	101	5
1	98	119	91	93	100	93	99	4
5	98	108	123	86	105	94	102	5
10	118	109	97	86	100	94	101	5
15	119	102	94	86	100	97	100	5
20	115	101	105	86	93	96	99	4

N = number of experiments (duplicated)

Table3.3 6 hr of incubation

%viability	% Control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
DMSO	106	98	104	95	107	90	100	3
0.1	104	83	103	95	115	89	98	5
0.5	94	97	109	94	110	97	100	3
1	106	101	99	95	108	91	100	3
5	97	103	108	95	112	91	101	3
10	103	102	100	97	110	99	102	2
15	87	87	84	79	90	80	85	2
20	84	87	83	100	85	78	86	3

N = number of experiments (duplicated)

Table 3.4 12 hr of incubation

%viability	% Control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
DMSO	96	103	94	93	95	89	95	2
0.1	116	115	91	96	92	99	102	4
0.5	100	99	100	88	96	89	95	2
1	105	99	99	94	101	91	98	2
5	99	107	99	93	84	99	97	3
10	97	109	101	93	91	94	98	3
15	79	83	90	81	77	80	82	2
20	78	84	88	80	77	86	82	2

Table3.5 24 hr of incubation

%viability	% Control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
DMSO	103	104	89	96	90	107	98	3
0.1	96	92	94	101	91	117	98	4
0.5	97	98	89	99	90	115	98	4
1	95	91	94	99	86	107	96	3
5	93	89	80	107	91	119	96	6
10	95	94	89	91	85	119	96	5
15	75	80	81	76	86	83	80	2
20	72	83	77	74	86	81	79	2

N = number of experiments (duplicated)

Table4 Effect of piperine on LDH release in cultured N1E-115 cells

Table4.1 1 hr of incubation

%viability	% Control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
DMSO	100	83	111	115	99	91	99	4
0.1	93	86	87	117	100	91	96	4
0.5	92	102	84	97	103	78	93	4
1	93	70	87	119	85	72	89	7
5	74	102	108	111	71	102	95	7
10	87	100	78	115	86	87	92	5
15	71	96	117	122	78	95	97	8
20	84	81	105	99	101	90	94	4

N = number of experiments (duplicated)

Table4.2 3 hr of incubation

%viability	% Control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
DMSO	98	86	93	94	99	120	98	4
0.1	94	81	76	96	108	132	97	7
0.5	107	96	87	115	96	113	102	4
1	90	97	89	103	98	129	100	6
5	98	117	92	103	88	127	102	6
10	101	96	100	109	93	112	101	3
15	99	110	87	94	106	119	101	4
20	99	94	109	97	104	138	105	6

N = number of experiments (duplicated)

Table4.3 6 hr of incubation

%viability	% Control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
DMSO	91	101	86	102	110	102	98	3
0.1	101	91	113	103	103	97	102	3
0.5	99	87	109	95	105	113	102	4
1	93	80	112	115	96	110	101	5
5	84	98	91	119	90	107	98	5
10	91	103	98	101	103	105	100	2
15	101	110	122	130	113	128	117	4
20	95	111	109	131	112	115	111	4

N = number of experiments (duplicated)

Table4.4 12 hr of incubation

%viability	% Control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
DMSO	90	101	116	105	102	79	98	5
0.1	102	93	129	108	87	88	100	6
0.5	100	91	100	101	92	83	94	3
1	91	93	121	95	83	86	94	5
5	103	79	122	114	94	91	100	6
10	89	96	111	107	96	82	96	4
15	113	110	129	116	105	100	111	4
20	110	108	119	115	107	100	109	2

N = number of experiments (duplicated)

Table4.5 24 hr of incubation

%viability	% control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
DMSO	115	87	98	84	87	122	98	6
0.1	101	92	96	86	87	117	96	4
0.5	107	84	102	91	99	115	99	4
1	102	101	86	99	87	119	98	4
5	117	86	102	92	102	122	102	5
10	120	82	100	82	106	115	100	6
15	124	107	91	105	106	125	109	5
20	122	105	107	99	110	136	112	5

N = number of experiments (duplicated)

Table5 Effect of piperine on H_2O_2 induced injuries in cultured N1E-115 cells determine by MTT reduction assay

Table5.1 Cotreatment for 6 hr

%viability	% Control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
Vehicle	107	104	115	94	112	104	106	3
H50	29	34	40	37	52	40	39	3
H+P.1	45	54	43	60	66	47	51	2
H+P.5	51	55	48	61	77	47	56	4
H+P1	66	68	43	56	79	34	58	6
H+P5	87	88	71	82	97	73	83	3
H+10	89	84	75	98	95	94	89	3

N = number of experiments (duplicated)

H, H50 : concentration of hydrogen peroxide equivalent to 50 μM

P.1,..P10 : concentration of piperine equivalent 0.1-10 μM respective

Table5.2 Pretreat with piperine for 12 hr incubation

%viability	% Control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
Vehicle	168	107	93	130	108	95	117	11
H50	46	49	45	54	45	41	47	4
H+P.1	38	64	33	80	39	38	49	8
H+P.5	35	59	46	45	58	28	45	5
H+P1	33	50	20	63	35	22	37	7
H+P5	31	41	33	43	36	18	34	4
H+10	33	31	17	34	30	16	27	3

N = number of experiments (duplicated)

Table5.2 Pretreat with piperine for 24 hr incubation

%viability	% Control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
Vehicle	96	103	92	116	112	95	102	4
H50	59	58	54	50	46	58	54	2
H+P.1	51	58	46	50	63	59	55	3
H+P.5	50	56	52	50	59	46	52	2
H+P1	56	58	61	45	51	57	55	2
H+P5	59	45	40	35	46	49	46	3
H+10	44	34	42	28	39	57	40	4

N = number of experiments (duplicated)

Table6 Effect of piperine on H_2O_2 induced injuries in cultured N1E-115 cells determine by LDH release assay

Table6.1 Cotreatment for 6 hr

%viability	% Control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
Vehicle	101	103	86	119	97	98	100	4
H50	124	127	124	144	128	136	131	3
H+P.1	131	129	106	135	121	133	125	4
H+P.5	123	130	95	130	128	102	118	6
H+P1	115	153	109	143	118	133	127	7
H+P5	108	156	97	108	97	106	111	8
H+10	106	151	106	110	104	104	112	7

N = number of experiments (duplicated)

Table6.2 Pretreat with piperine for 12 hr incubation

%viability	% Control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
Vehicle	82	112	103	96	120	99	101	5
H50	116	154	124	125	156	130	133	6
H+P.1	124	133	131	127	150	130	132	3
H+P.5	125	165	121	130	155	131	136	7
H+P1	123	137	145	131	169	131	138	6
H+P5	127	131	145	127	134	129	132	2
H+10	127	134	144	126	161	131	136	5

N = number of experiments (duplicated)

Table6.2 Pretreat with piperine for 24 hr incubation

%viability	% Control							
Concentration(μM)	N1	N2	N3	N4	N5	N6	Mean	S.E.M
Control	100	100	100	100	100	100	100	0
Vehicle	87	111	127	84	97	110	101	6
H50	134	137	153	118	129	147	134	5
H+P.1	124	130	131	106	117	161	126	7
H+P.5	149	117	163	124	125	145	136	7
H+P1	126	142	144	124	125	148	133	4
H+P5	129	121	156	127	120	151	132	6
H+10	128	146	159	111	122	144	133	7

N = number of experiments (duplicated)

BIOGRAPHY

NAME	Mr. Prasit Opas
DATE OF BIRTH	January 7, 1974
PLACE OF BIRTH	Ubon Ratchathani Province, Thailand
INSTITUTION ATTENDED	Bachelor of Science in Pharmacy Khon Kaen University, 1997 Bachelor of Laws Sukhothai Thummathirat University, 2003
POSITION & OFFICE	Pharmacist Bureau of Drug and Narcotic, Department of Medical Science, Ministry of Public Health Nonthaburi, Thailand Tel. (02) 9510000 (99112)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย