การได้รับยาพาราเซตามอลอย่างเรื้อรังในหนูแรทสามารถเหนี่ยวนำให้มีการเพิ่มขึ้นของสาร pro-inflammatory cytokines เมื่อถูกกระตุ้นด้วยปรากฏการณ์ cortical spreading depression

นางสาวฉัตรพร ฉันท์ทอง

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CHRONIC PARACETAMOL TREATMENT INDUCES AN INCREMENT OF PRO-INFLAMMATORY CYTOKINES IN RATS WITH CORTICAL SPREADING DEPRESSION

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ฉัตรพร ฉันท์ทอง : การได้รับยาพาราเซตามอลอย่างเรื้อรังในหนูแรทสามารถเหนี่ยวนำให้มีการเพิ่มขึ้นของสาร pro-inflammatory cytokines เมื่อถูกกระตุ้นด้วยปรากฏการณ์ cortical spreading depression. (CHRONIC PARACETAMOL TREATMENT INDUCES AN INCREMENT OF PRO-INFLAMMATORY CYTOKINES IN RATS WITH CORTICAL SPREADING DEPRESSION) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. ศุภางค์ มณีศรี เลอกรองด์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ. ดร. ธนัญญา ทองตัน. 83 หน้า

การศึกษาครั้งนี้มีจุดประสงค์เพื่อศึกษาผลของการได้รับยาพาราเซตามอลอย่างเรื้อรังต่อการเปลี่ยนแปลงของระดับสาร pro-inflammatory cytokines บริเวณผิวสมอง เมื่อถูกกระตุ้นด้วยปรากฏการณ์คอร์ติคัลสเปรดดิ้งดีเพรสชั่น โดยแบ่งหนูทดลอง (น้ำหนัก 250-300 กรัม) ออกเป็น 4 กลุ่ม คือ กลุ่มควบคุม กลุ่มควบคุมที่ได้รับการกระตุ้นให้เกิดปรากฏการณ์คอร์ติคัลสเปรดดิ้งดีเพรสชั่น กลุ่มที่ได้รับยาพาราเซตามอลอย่างเดียว และกลุ่มที่ได้รับยาพาราเซตามอลร่วมกับการกระตุ้นให้เกิดปรากฏการณ์คอร์ติคัลสเปรดดิ้งดีเพรสชั่น (แต่ละกลุ่ม n=10) กลุ่มที่ได้รับยาพาราเซตามอลไม่ว่าจะเป็นกลุ่มที่ได้รับหรือไม่ได้รับการกระตุ้นให้เกิดปรากฏการณ์คอร์ติคัลสเปรดดิ้งดีเพรสชั่นจะได้รับการฉีดยาเข้าทางช่องท้อง (200 มิลลิกรัมต่อน้ำหนักตัว 1 กิโลกรัม) ทุกวันเป็นระยะเวลาต่างๆ กันดังนี้ คือ 0, 5, 15 และ 30 วัน ขณะที่กลุ่มที่ไม่ได้รับยาพาราเซตามอลจะได้รับการฉีดด้วย 0.9% normal saline เมื่อครบกำหนดเวลา หนูทุกตัวจะได้รับการฉีดยาสลบก่อนทำการเก็บตัวอย่างเพื่อนำไปศึกษาการแสดงออกของระดับสารที่เกี่ยวข้องกับการอักเสบ (IL-1α และ TNF-α) โดยวิธี immunohistochemistry และ western blot analysis นอกจากนั้นยังทำการศึกษา signaling pathway ที่เกี่ยวข้องกับกระบวนการอักเสบ (NF-kB, phospho-NF-kB และ phospho-IkB) โดยวิธี western blot analysis ด้วย

ผลการศึกษาพบว่า การได้รับยาพาราเซตามอลเป็นระยะเวลาสั้น (0 และ 5 วัน)ไม่ว่าจะเป็นกลุ่มที่ได้รับยาพาราเซตามอลอย่างเดียวหรือ ร่วมกับการกระตุ้นด้วยปรากฏการณ์คอร์ติคัลสเปรดดิ้งดีเพรสชั่น ไม่มีผลต่อการเปลี่ยนแปลงของระดับ IL-1α และ TNF-α นอกจากนั้น การศึกษาวิจัยในกลุ่มที่ได้รับการกระตุ้นด้วยปรากฏการณ์คอร์ติคัลสเปรดดิ้งดีเพรสชั่นเพียงอย่างเดียวโดยไม่ได้รับยาพาราเซตามอลร่วมด้วย ก็พบว่า ไม่มีผลต่อการเปลี่ยนแปลงของระดับ IL-1α และ TNF-α แต่อย่างไรก็ตามผลของการศึกษาในกลุ่มที่ได้รับยาพาราเซตามอลอย่างเรื้อรัง (15 และ 30 วัน) ทั้งกลุ่มที่ได้รับและไม่ได้รับการกระตุ้นด้วยปรากฏการณ์คอร์ติคัลสเปรดดิ้งดีเพรสชั่น พบว่า มีการเพิ่มขึ้นของจำนวนเซลล์ที่มีการหลั่ง IL-1α และ TNF-α ซึ่งสอดคล้องกับการศึกษาระดับของ IL-1α และ TNF-α ด้วยเทคนิค western blot เมื่อเปรียบเทียบระหว่างกลุ่มที่ได้รับยาพาราเซตามอลเรื้อรังเพียงอย่างเดียวกับกลุ่มที่ได้รับยาพาราเซตามอลเรื้อรังร่วมกับการกระตุ้นด้วยปรากฏการณ์คอร์ติคัลสเปรดดิ้งดีเพรสชั่น พบว่า ในกลุ่มที่ได้รับยาพาราเซตามอลเรื้อรังร่วมกับการกระตุ้นด้วยปรากฏการณ์คอร์ติคัลสเปรดดิ้งดีเพรสชั่นมีระดับของ IL-1α และ TNF-α มากกว่า นอกจากนี้ยังพบว่า ทั้งในกลุ่มที่ได้รับยาพาราเซตามอลเรื้อรังเพียงอย่างเดียวและกลุ่มที่ได้รับยาพาราเซตามอลเรื้อรังร่วมกับการกระตุ้นด้วยปรากฏการณ์คอร์ติคัลสเปรดดิ้งดีเพรสชั่น (15 และ 30 วัน) มีการเปลี่ยนแปลงระดับของ phospho-NF-kB และ phospho-IkB ที่เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุม ซึ่งเป็นการเปลี่ยนแปลงไปในทิศทางเดียวกับการเพิ่มขึ้นของระดับสารหลั่งที่เกี่ยวข้องกับการอักเสบ

จากการศึกษาดังกล่าวแสดงให้เห็นว่าการได้รับยาพาราเซตามอลอย่างต่อเนื่องเป็นระยะเวลานานสามารถเพิ่มระดับของสารหลั่งที่เกี่ยวข้องกับการอักเสบได้ โดยการเปลี่ยนแปลงที่เกิดขึ้นนั้นน่าจะมีความเกี่ยวข้องกับการกระตุ้นผ่านวิถี NF-kB signaling

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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CHATTRAPORN CHANTONG : CHRONIC PARACETAMOL TREATMENT INDUCES AN INCREMENT OF PRO-INFLAMMATORY CYTOKINES IN RATS WITH CORTICAL SPREADING DEPRESSION. ADVISOR: ASST. PROF. SUPANG MANEESRI-LE GRAND, Ph.D., CO-ADVISOR: ASST. PROF.THANANYA THONGTAN, Ph.D, 83 pp.

The present study aimed to investigate the effect of chronic treatment with paracetamol on the production of pro-inflammatory cytokines IL-1α and TNF-α in the cerebral cortex. Male Wistar rats (250-300 g) were divided into 4 groups: the control, control with CSD activation, paracetamol, and paracetamol with CSD activation group (n=10, each). A single dose and daily paracetamol (200 mg/kg BW, intraperitonealy) treatment for a period of 0, 5, 15, and 30 days was injected into the rats of the paracetamol treated group whereas a vehicle was injected into those of the control group at the same volume. After completion of the treatment, all rats were humanely killed by injection of an excessive dose of sodium pentobarbital. The expression of pro-inflammatory cytokines (IL-1α/TNF-α) was studied by immunohistochemistry and western blot analysis. Since the transcription factor of the nuclear factor kB (NF-kB) is an important signaling pathway involved in the inflammatory responses, the phosphorylation of NF-kB and IkB were studied by western blot analysis in all experimental groups as well.

The results showed that short term exposure or acute paracetamol treatment at periods of 0 and 5 days had no effect on the expression of pro-inflammatory cytokines IL-1α and TNF-α. The CSD activation without paracetamol treatment had no effect on the expression of both cytokines. However, long term exposure or chronic paracetamol treatment in both combination with and without CSD activation for 15 and 30 days induced an increase in the number of IL-1 α and TNF-α immunoreactive cells in the cerebral cortex more than those observed in the control group and correlated with the expression of these proteins level by western blot analysis. When compared between the chronic paracetamol with and without CSD activation, the expression of those cytokines in the paracetamol with CSD activation was higher. In addition, the expression of phospho-NF-kB and phospho-IkB in the rat with chronic paracetamol treatment (with and without CSD activation) for 15 and 30 days were significantly enhanced when compared with control group.

The results of the present study suggest that chronic paracetamol treatment leads to an increase in the pro-inflammatory cytokines (both IL-1α and TNF-α) production and the enhancement of those cytokines might mediate via the activation of the NF-kB signaling pathway.

Field of Study : Medical Sciences Student's Signature .....

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Co-advisor's Signature .....

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CONTENTS

	Page
ABSTRACT IN THAI	iv
ABSTRACT IN ENGLISH	v
ACKNOWLEDGEMENTS	vi
CONTENTS	vii
LIST OF TABLES	x
LIST OF FIGURES	xi
LIST OF ABBREVIATIONS	xiv
CHAPTER I INTRODUCTION	1
1.1 Background and rationale	1
1.2 Research questions	3
1.3 Objectives	3
1.4 Hypothesis	3
1.5 Key words	4
1.6 Research design	4
1.7 Expected benefits and application	4
CHAPTER II LITERATURE REVIEW	5
2.1 Paracetamol	6
2.1.1 Paracetamol and mechanisms of action	6
2.1.2 Metabolism of paracetamol	8
2.1.3 Effect of paracetamol treatment	10
2.1.4 Paracetamol and Inflammation	11
2.2 Cortical spreading depression (CSD)	12
2.2.1 CSD and neurogenic inflammation	12

Page

2.3 Pro-inflammatory cytokines 13
2.3.1 Interleukin-1 alpha (IL-1α) 13
2.3.2 Tumor necrosis factor-alpha (TNF-α) 13
2.4 NF-kB signaling pathway 15
CHAPTER III METHODOLOGY 17
3.1 Animals 17
3.2 Study design 17
3.3 Methods 20
3.3.1 Surgical operation and induction of CSD 20
3.3.2 Sample collection 21
3.3.2.1 Perfusion and tissue preparation 21
3.3.2.2 Preparation of Fresh frozen tissue 21
3.4 Liver function test 21
3.4.1 Liver enzyme levels and histological analysis 21
3.5 Immunohistochemical study 22
3.6 Western blot analysis 23
3.7 Data analysis 24
CHAPTER IV RESULTS 25
4.1 The effect of chronic paracetamol treatment on the hepatotoxicity 25
4.2 The effect of chronic paracetamol treatment on the CSD-induced the expression of interlukin-1 alpha (IL-1α) in the cerebral cortex 27
4.3 The effect of chronic paracetamol treatment on the CSD-induced the expression of tumor necrosis factor-alpha (TNF-α) in the cerebral cortex 36
4.4 The effect of chronic paracetamol treatment on the CSD-induced the activation of the NF-kB signaling pathway 45
4.4.1 Expression of NF-kB 45
4.4.2 Expression of phospho-NF-kB 55
4.4.3 Expression of phospho-IkB 60
4.5 Conclusion of results 65

Page

CHAPTER V DISCUSSION 67

CHAPTER VI CONCLUSION 72

REFERENCES 73

APPENDIX 78

BIOGRAPHY 83

LIST OF TABLES

Table		Page
4-1	Theeffect of chronic paracetamol treatment at four different time points (0, 5, 15, and 30 days) on the alanine aminotransferase (AST), aspartate aminotransferase (ALT), and alkaline phosphatase (ALP) levels in rats serum	26
4-2	Effect of paracetamol treatment for four different time points on the pro-inflammatory cytokine IL-1α expression in the rat's cerebral cortex	35
4-3	Effect of paracetamol treatment for four different time points on the pro-inflammatory cytokine TNF-α expression in the rat's cerebral cortex	44
4-4	Effect of paracetamol treatment at four different time points on the expression of transcription factor NF-kB in the rat's cerebral cortex	54

LIST OF FIGURES

Figure		Page
2-1	Stracture of paracetamol	6
2-2	Pathway of paracetamol metabolism	9
2-3	Overview of the TNFR1 signaling pathway	14
2-4	NF-kB signaling pathway	16
3-1	Study design of this experiment	18
4-1	The effect of (a) 0 days and (b) 5 days paracetamol treatment on CSD-induced an expression of IL-1α in the cerebral cortex detected by western blotting	28
4-2	The effect of (a) 0 days and (b) 5 days paracetamol treatment on the IL-1α expression in the brain. The photomicrographs showing the expression of the IL-1α immunoreactive cells in the cerebral cortex	29
4-3	The effect of 15 days paracetamol treatment on CSD-induced an expression of IL-1α in the cerebral cortex	31
4-4	The photomicrograph shows localization of the IL-1α immunoreactive cells in the cerebral cortex at 15 days	32
4-5	The effect of 30 days paracetamol treatment on CSD-induced an expression of IL-1α in the cerebral cortex	33
4-6	The photomicrograph shows localization of the IL-1α immunoreactive cells in the cerebral cortex at 30 days	34
4-7	The effect of (a) 0 days and (b) 5 days paracetamol treatment on CSD-induced an expression of TNF-α in the cerebral cortex detected by western blotting	37
4-8	The effect of (a) 0 days and (b) 5 days paracetamol treatment on the TNF-α expression in the brain. The photomicrographs showing the expression of the TNF-α immunoreactive cells in the cerebral cortex	38
4-9	The effect of 15 days paracetamol treatment on CSD-induced an expression of TNF-α in the cerebral cortex	40

Figure **Page**

4-10 The photomicrograph shows localization of the TNF-α immunoreactive cells in the cerebral cortex at 15 days 41

4-11 The effect of 15 days paracetamol treatment on CSD-induced an expression of TNF-α in the cerebral cortex 42

4-12 The photomicrograph shows localization of the TNF-α immunoreactive cells in the cerebral cortex at 30 days 43

4-13 The effect of 0 days paracetamol treatment on CSD-induced an expression of the transcription factor NF-kB p65 in the cerebral cortex 46

4-14 The photomicrograph shows localization of the NF-kB immunoreactive cells in the cerebral cortex at 0 days 47

4-15 The effect of 5 days paracetamol treatment on CSD-induced an expression of the transcription factor NF-kB p65 in the cerebral cortex 48

4-16 The photomicrograph shows localization of the NF-kB immunoreactive cells in the cerebral cortex at 5 days 49

4-17 The effect of 15 days paracetamol treatment on CSD-induced an expression of the transcription factor NF-kB p65 in the cerebral cortex 50

4-18 The photomicrograph shows localization of the NF-kB immunoreactive cells in the cerebral cortex at 15 days 51

4-19 The effect of 30 days paracetamol treatment on CSD-induced an expression of the transcription factor NF-kB p65 in the cerebral cortex 52

4-20 The photomicrograph shows localization of the NF-kB immunoreactive cells in the cerebral cortex at 30 days 53

4-21 The effect of 0 days paracetamol treatment on CSD-induced an expression of the phospho-NF-kB p65 in the cerebral cortex 56

4-22 The effect of 5 days paracetamol treatment on CSD-induced an expression of the phospho-NF-kB p65 in the cerebral cortex 57

4-23 The effect of 15 days paracetamol treatment on CSD-induced an expression of the phospho-NF-kB p65 in the cerebral cortex 58

Figure **Page**

4-24 The effect of 30 days paracetamol treatment on CSD-induced an expression of the phospho-NF-kB p65 in the cerebral cortex 59

4-25 The effect of 0 days paracetamol treatment on CSD-induced an expression of phospho-IkB in the cerebral cortex 61

4-26 The effect of 5 days paracetamol treatment on CSD-induced an expression of phospho-IkB in the cerebral cortex 62

4-27 The effect of 15 days paracetamol treatment on CSD-induced an expression of phospho-IkB in the cerebral cortex 63

4-28 The effect of 30 days paracetamol treatment on CSD-induced an expression of phospho-IkB in the cerebral cortex 64

4-29 The expression of IL-1α and TNF-α in the cerebral cortex in four different time points 65

LIST OF ABBREVATIONS

ALP	=	alkaline phosphatase
ALT	=	aspartate aminotransferase
AST	=	alanine aminotransferase
APAP	=	acetaminophen
BBB	=	blood-brain barrier
BW	=	body weight
BSA	=	bovine serum albumin
CBF	=	cerebral blood flow
CGRP	=	calcitonin gene related peptide
COX	=	cyclooxygenase
CSD	=	cortical spreading depression
CNS	=	central nervous system
CYP2E1	=	cytochrome P450 2E1
DAB	=	3, 3-diaminobenzidin
GSH	=	glutathione
KCl	=	potassium chloride
IkB	=	inhibitor of kappa B
IKK	=	I kappa B kinase
IL-1α	=	interlukin-1 alpha
NaCl	=	sodium chloride
NAPQI	=	*N*-acetyl-*p*-benzoquinone imine
NSAIDs	=	non-steroidal anti-inflammatory drugs
NO	=	nitric oxide
NF-kB	=	nuclear factor-kappa B
NMDA	=	N-methyl-D-Aspartate
MAP	=	mitogen-activated protein
MCP-1	=	monocyte chemoattractant protein-1
MIP-1α	=	macrophage inhibitory protein-1 alpha

PBS	=	phosphate buffer saline
PGHS	=	prostaglandin H_2 synthetase
POX	=	peroxidase site
SP	=	substance P
TBS	=	tris-buffered saline
TNC	=	trigeminal nucleus caudalis
TNF-α	=	tumor necrosis factor-alpha
5-HT	=	serotonin
µm	=	micrometer
kg	=	kilogram
mg	=	milligram
mm	=	millimeter
i.p.	=	intraperitoneal

CHAPTER I

INTRODUCTION

1.1 Background and rationale

Paracetamol (acetaminophen, APAP) is the most widely used over-the-counter medication as an antipyretic and analgesic drug [1]. This drug became one of the most popular drugs used for treatment of pain and several types of headaches because of various properties of this drug including low price, high availability without prescription and low impact of side effect. Even though this drug has been distributed for several decades (since 1953), its mechanism of action is still controversial. Several mechanisms by which paracetamol achieves its analgesic effect have been proposed including the alteration of the descending serotonergic anti nociceptive system [2], inhibition on the spinal NO pathway [3], modulation of the endogenous cannabinoid system, and anti-inflammatory action by inhibition of cyclooxygenase (COX) [4].

Paracetamol became one of the most popular drugs used for treatment of pain and several types of headaches including migraine. It is often recommended to be used as the first line of treatment for an acute migraine attack. Paracetamol has long been considered as a safe drug. Several lines of evidence have indicated the protective effect of paracetamol on several pathological conditions [1, 5-6]. With therapeutic doses, the side effect of this drug is limited. Hepatotoxicity is the most frequent adverse effect reported after acute overdoses and chronic treatment with higher doses than that of therapeutic range [7]. However, few recent studies have reported the adverse effect of this drug on other organs including the CNS. The recent study by *Posadas et al.* has demonstrated that paracetamol at the doses below those required to produce hepatotoxicity could produce the neurotoxic effect both *in vitro* and *in vivo* studies [8]. Furthermore, the studies in the cortical spreading depression (CSD) animal headache model have demonstrated that chronic treatment with paracetamol could upregulate the 5-HT_2 receptors in the brain. The increase of this receptor occurred in parallel with the increase in the cortical neuron activity as well as the trigeminovascular nociceptive responses [9-11]. Their results indicated that the chronic treatment of paracetamol might sensitize the trigeminovascular nociception in both

the peripheral and central pathway. However, the mechanism underlying the deterioration of the trigeminal nociceptive system has not been clarified.

It is known that the neurogenic inflammation is one of the mechanisms which can sensitize the trigeminovascular nociceptive system. Paracetamol, despite being classified as a non-steroidal anti-inflammatory drugs (NSAIDs), demonstrates the dual effect in inflammatory processes. At a high dose, it can induce a series of both pro- and anti-inflammatory cytokines [1, 12]. Thus, it might be possible that the chronic treatment with paracetamol induces the alteration in inflammatory response to CSD and leads to the sensitization of the trigeminovascular nociceptive system. In order to clarify this point, with this study we aimed to investigate the effect of chronic paracetamol treatment (200 mg/kg BW i.p.) on the production of pro-inflammatory cytokines in the rat brain with and without the activation of cortical spreading depression (CSD). The effect of paracetamol treatment on the pro-inflammatory cytokines were studied in four different time points (0, 5, 15, and 30 days). The expression of IL-1α and TNF-α were studied by western blot analysis and immunohistochemistry. Since transcription factor of the nuclear factor kB (NF-kB) is an important signaling pathway involved in the inflammatory responses. The phosphorylation of IkB (the inhibitor of kB) and the activation of NF-kB were examined using western blotting in all experimental groups as well.

1.2 Research questions

Can chronic paracetamol treatment induce an increase in pro-inflammatory cytokine production in rat brains with CSD activation?

1.3 Objectives

Main objective:

1. To determine the effect of 30 days paracetamol treatment on the production of pro-inflammatory cytokines in rat brains using the cortical spreading depression (CSD) model.

Minor objectives:

1. To determine the effect of 15 days paracetamol treatment on the production of pro-inflammatory cytokines in rat brains using the cortical spreading depression (CSD) model.
2. To determine the effect of 5 days paracetamol treatment on the production of pro-inflammatory cytokines in rat brains using the cortical spreading depression (CSD) model.
3. To determine the effect of 0 day paracetamol treatment on the production of pro-inflammatory cytokines in rat brains using the cortical spreading depression (CSD) model.

1.4 Hypothesis

Chronic paracetamol treatment can increase the production of pro-inflammatory cytokines in rat brains with CSD activation

1.5 Key words

Paracetamol, Pro-inflammatory cytokines, Cortical Spreading Depression, NF-kB signaling

1.6 Research design

Animal experimental design

1.7 Expected benefits and application

The findings will be relevant in understanding the effect of chronic paracetamol treatment on the production of pro-inflammatory cytokines in the rat's brain.

CHAPTER II

REVIEW OF LITERATURE

2.1. Paracetamol

2.1.1 Paracetamol and mechanisms of action

2.1.2 Metabolism of paracetamol

2.1.3 Effect of paracetamol treatment

2.1.4 Paracetamol and Inflammation

2.2. Cortical spreading depression (CSD)

2.2.1 CSD and neurogenic inflammation

2.3. Pro-inflammatory cytokines

2.3.1 Interleukin-1 alpha (IL-1α)

2.3.2 Tumor necrosis factor-alpha (TNF-α)

2.4. NF-kB signaling pathway

2.1 Paracetamol

Paracetamol (acetaminophen, APAP), as an antipyretic and analgesic drug, is one of the most widely used over-the-counter medications. Paracetamol is considered as a safe drug. The most frequently reported adverse effect is hepatotoxicity when taken as an acute and chronic overdose [8]. Even though this drug is distributed for more than 60 years, the mechanisms of action are still unclear [4, 9].

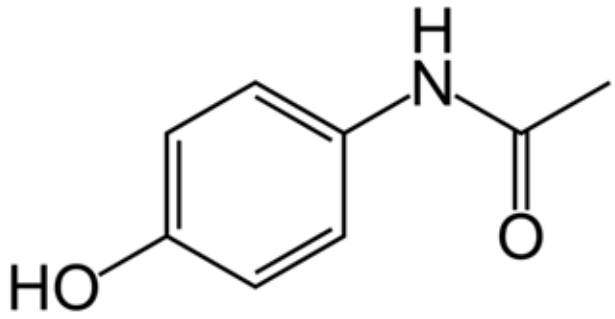


Figure 2-1 Stracture of paracetamol

2.1.1 Paracetamol: mechanisms of actions

Based on several reviews, the proposed mechanisms of action of this drug are as follows:

A central serotonergic mechanism

Several studies have indicated that the modulation of the central serotonin system has been involved in the anti-nociceptive effect of paracetamol. The result obtained from the study in animals has confirmed that the 5-hydroxytryptamine type 3 (5-HT_3) receptors are involved in the analgesic effect of paracetamol [13-14]. The data obtained from the study in humans have confirmed this hypothesis. They found that co-administration of tropisetron or granisetron (5-HT_3 receptor antagonists) with paracetamol could abolish the analgesic effect in volunteers [15]. Based on these data, it can be suggested that paracetamol can reinforce the serotonin descend in the inhibitory pain pathways which results in the anti-nociceptive effect of this drug [16].

Prostaglandin H_2 synthetase inhibition

The inhibition of prostaglandin synthesis is an alternative mechanism which explains the anti-nociceptive effect of paracetamol. It is known that the prostaglandin H_2 synthetase (PGHS) is the enzyme responsible for the metabolism of arachidonic acid to the PGH2. This enzyme is commonly referred to as COX [17]. It has two isoforms which are the constitutive PGHS-1(COX-1) and the inducible PGHS-2 (COX-2). There are two active sites on PGHS: a cyclooxygenase site (COX) and a peroxidase site (POX). Several studies have suggested that paracetamol reduced the amount of the oxidized form of the COX enzyme by an action on the POX site [2-3, 18]. In addition, the study by Chandrasekharan et al. have suggested that paracetamol could inhibit the other PGHS variant (COX-3) which exists in a high concentration in the central nervous system (CNS) [19]. The study in the dog brain by Flower and Vane demonstrated that paracetamol acting centrally by inhibiting the cyclooxygenase (COX) activity in the brain homogenates more than those from spleen [20]. However, the hypothesis of COX-3 inhibition has not been confirmed by experimental evidence in humans [19]. Taken together, it can be suggested that the inhibition of prostaglandin synthesis might be at least one mechanism underlying the anti-nociceptive and anti-pyretic effect of paracetamol.

Nitric oxide

The results obtained from the study in animals suggest that depolarization of afferent neurons by peripheral harmful stimuli leads to an activation of spinal N-methyl-D-Aspartate (NMDA) receptors and promote the synthesis of nitric oxide (NO); a neurotransmitter at a spinal level conveying nociceptive information. Paracetamol and NSAIDs involvement in the nociceptive process occurs at the spinal level by inhibition of the NMDA receptor activation. This effect may associate with an inhibition of NO mechanisms [21].

2.1.2 Metabolism of paracetamol

Paracetamol was absorbed rapidly and its plasma half-life is about 2 hours. This drug is mainly metabolized in the liver via conjugation with glucuronic acid (60%), sulphuric acid (30%) or cysteine (3%) and then excreted in the urine as a conjugated form. A small fraction of this drug (10%) is metabolized by cytochrome P-450 enzymes to form a reactive metabolite, n-acetyl-p-benzoquinone imine (NAPQI). The most important isoform of cytochrome P-450 for the oxidative pathway is CYP2E1. The highest concentration of this enzyme is expressed by the liver. However, accumulative evidences have demonstrated that the enzyme CYP2E1 expression was also found in the lung, the kidney, and the brain [22]. Normally, after forming the NAPQI, it will be rapidly detoxify by glutathione (GSH) and these non-toxic glutathione conjugated will finally excrete in the urine [23, 24].

In case of overdose ingestion of paracetamol, hepatic GSH storage will be depleted, the free NAPQI bind to cysteine groups on cellular proteins to form paracetamol-protein adducts. Binding of NAPQI with mitochondrial proteins can lead to hepatocellular death. [23, 25].

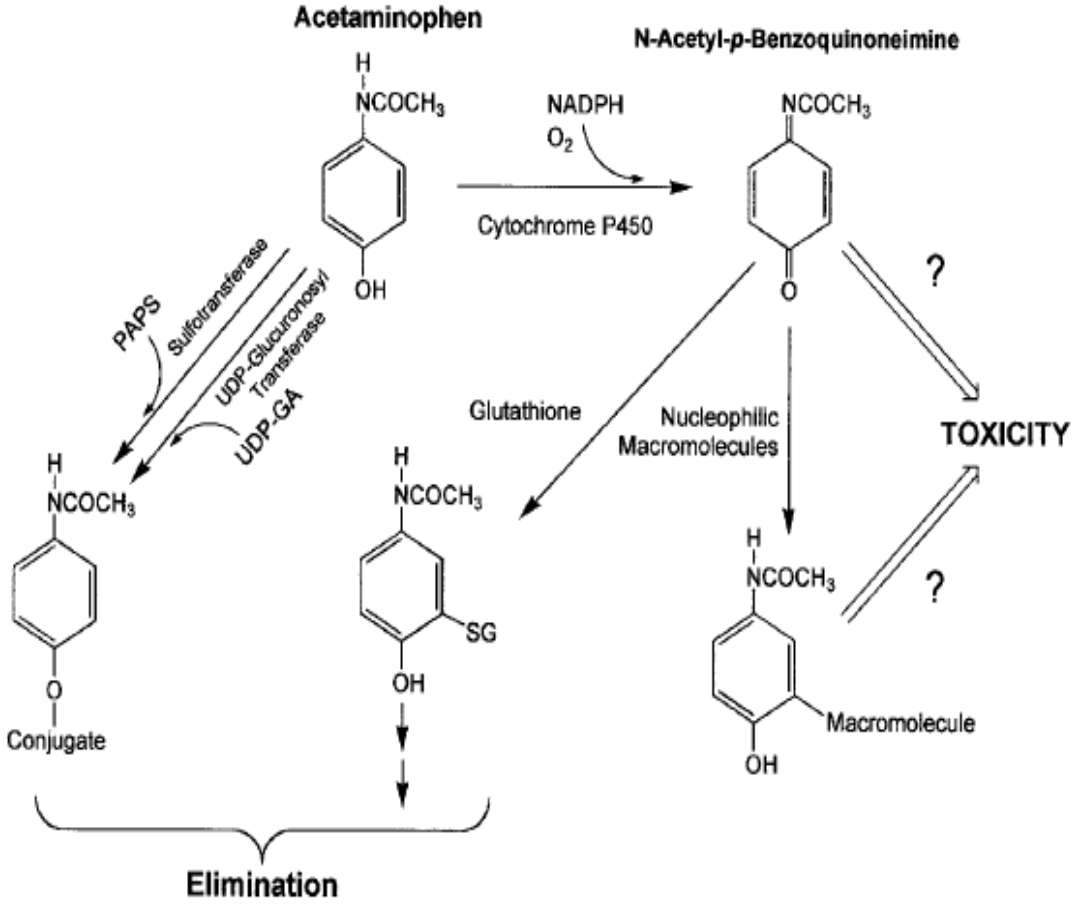


Figure 2-2 Pathway of paracetamol metabolism [23]

2.1.3 Effect of paracetamol treatment

Most of the reports related to the effect of paracetamol are about the protective effect. In 2009, it was reported that pre-treatment cultured of brain endothelial cells with paracetamol could decrease the pro-inflammation cytokine production (IL-1α, IL-6, TNF-α) induced by an oxidant stressor (menadione). They concluded that paracetamol could protect brain endothelial cells against oxidative stress [1]. They also found that pre-treatment of cultured neurons with paracetamol could inhibit neuronal inflammation and protect neurons from oxidative stress [5]. The study in the transient global forebrain ischemia model has suggested that paracetamol could reduce tissue damage, the degree of mitochondrial swelling, and the loss of mitochondrial outer membrane permeabilization which indicates that paracetamol can reduce the apoptosis via a mitochondrial–mediated mechanism [6]. Based on their results, the therapeutic role of paracetamol on the stroke treatment has been suggested.

Even though the accumulative data have indicated the protective role of paracetamol, the adverse effects of this drug on several organs have recently reported. It was reported that 4 weeks treatment with paracetamol (200 mg/kg twice a day) could induce serum sulfate depletion [26]. In 2000, in vitro study in cultured pneumocytes had demonstrated that after 4 hours incubation with paracetamol (0.05-1 mM) the intracellular glutathione level in pneumocytes had decreased. They suggested that the decrease of the glutathione level might cause an increase in the oxidative stress which could be a risk factor for asthma [27]. A more recent study in 2010 has demonstrated that paracetamol could induce apoptosis in rat cortical neurons both in vitro and in vivo experiments. Their results demonstrated that paracetamol at the concentration of 1 and 2 mM caused direct neuronal death. The same result was observed when paracetamol at the doses of 250 and 500 mg/kg i.p. were injected into the rats [8]. The alteration of the trigeminovascular nociceptive system after chronic treatment with paracetamol had been reported in 1999. Rats with chronic paracetamol treatment (dose 400 mg/kg i.p. for 30 days) demonstrated a decrease in tail flick latency in the hot plate test [9]. Recent studies in the CSD animal headache model have demonstrated that chronic paracetamol treatment (dose 200 mg/kg i.p. for 30 days) could enhance the CSD-induced trigeminovascular nociception. The increase of trigeminal nociception had occurred in parallel with the hyperexcitability of the cortical neurons and the increase in the 5-HT_{2A} receptor in the brain [10-11].

2.1.4 Paracetamol and Inflammation

Despite being classified as a non-steroidal anti-inflammatory drug, the anti-inflammatory action of paracetamol is not fully understood. At a high dose, the administration of paracetamol initiates a series of both pro- and anti-inflammatory cascades. It was reported that paracetamol at the dosage of 300 mg/kg i.p. could induce an increase in the expression of pro-inflammatory mediators, interleukin (IL-1β), tumor necrosis factor alpha (TNF-α), macrophage inhibitory protein-1 alpha (MIP-1α), monocyte chemoattractant protein-1 (MCP-1), as well as the anti-inflammatory cytokines IL-10 and IL-13 [1, 12].

At a low dose, paracetamol can protect neuronal and brain endothelial cells from oxidative stress and inhibit inflammation. The results from several studies have demonstrated that the expression of inflammatory mediators by neuronal and brain endothelial cells were increased in response to oxidative stress and that pre-treatment of those cells with paracetamol could decrease the inflammatory protein expression (RANTES, MIP-1α, TNF-α, IL-1α, IL-1β) [1, 5]. Based on these accumulative data, it can be suggested there should be other, as yet unidentified biochemical targets for both the toxic and therapeutic actions of acetaminophen.

2.2 Cortical spreading depression (CSD)

Cortical spreading depression was discovered over 60 years ago by Leào [28]. CSD is a propagating wave of neuronal and glial depolarization which can occur in several parts of the brain including the cerebral cortex, hippocampus, and cerebellum [29]. CSD transverses the cerebral cortex at a velocity of 2-3 mm/min. CSD has been implicated in several neurovascular disorders including stroke and migraine. CSD can be induced experimentally in animals by the topical application of potassium chloride (KCl) to the cortical surface. Induction of CSD can lead to the alteration in both neural and vascular compartments [30]. It has been reported to associate with the alteration in extracellular levels of ions [31], neurotransmitters [32], metabolic rate [33], cerebral blood flow (CBF), and pro-inflammatory cytokines [34].

2.2.1 CSD and neurogenic inflammation

CSD can induce the sterile neurogenic inflammation by stimulating the trigeminal ganglion which results in the neuropeptides release for example the calcitonin gene related peptide (CGRP) and substance P (SP) from trigeminocervical nerve endings. The increased level of these neuropeptides can evoke a cascade of events characterized by vasodilatation, plasma protein extravasation, and the release of several pro-inflammatory mediators such as TNF-α and IL-1β [34]. These phenomena can finally lead to neurogenic inflammation. Neurogenic inflammation can activate the trigeminal vascular nociceptive system and cause a headache [30]. The involvement of these neurogenic inflammations in migraine pathophysiology has been confirmed by the result from clinical study which demonstrated that intravenously injection of sumatriptan which can inhibit the neuropeptides release can decrease the pain score in migraine patients [35].

2.3 Pro-inflammatory cytokines

2.3.1 Interleukin-1 alpha (IL-1α)

Interleukin-1 alpha (IL-1α) is a pro-inflammatory cytokine that has been implicated in a variety of pathophysiological processes. [36]. IL-1, a general name for two distinct isoforms, IL-1α and IL-1β, mediate the biological activities and bind to the same cell surface receptor. Within the two distinct isoforms, IL-1 was the cytokine identified with actions on the brain. Upregulation of IL-1 in the meninges appears to play a key role in the induction of the inflammatory response produced by brain activation [30]. The results from clinical studies in children with migraine attacks have shown that the level of plasma IL-1α was undetectable in control subjects with tension-type headache, however, in patients with migraine this cytokines could be detectable, which indicates that the IL-1α might be involved in migraine pathophysiology [37]. Binding of IL-1 agonists to the IL-1 receptor (IL-1R) can lead to the activation of nuclear factor-kappa B (NF-kB) and mitogen-activated protein (MAP) kinase pathways [38].

2.3.2 Tumor necrosis factor-alpha (TNF-α)

Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine produced by T cells and cells of the monocyte/macrophage lineage, including microglial cells [39]. Molecular studies have demonstrated that two types of TNF-α are produced as 26 kDa precursor and secreted as 17 kDa. Several studies have indicated that TNF-α is involved in many pathological conditions in CNS including neurodegenerative disease and migraine [39]. At the beginning microglia was found to be a cell which produces TNF-α [40]. However, it is well established now that apart from the microglial cells, TNF-α can also be synthesized and released in the brain by astrocytes and some populations of neurons. There are two specific receptors for TNF-α: TNFR1 and TNFR2. Both TNF-α receptors are expressed by neurons and glia in the brain. The expression of these receptors can vary based on the cellular response to the apoptosis or inflammation. TNFR1 may result in activation of apoptosis or transcriptional activity. The role of

TNFR2 in the brain is not well established [39]. TNF-α after binding with its receptor will result in the recruitment of several adapter proteins including : RIP is a protein kinase which plays a critical role in activation of IKK for which phos-phorylate I-kB leads to the dissociation of the I-kB/NF-kB complex and nuclear translocation of active transcription factor NF-kB [41].

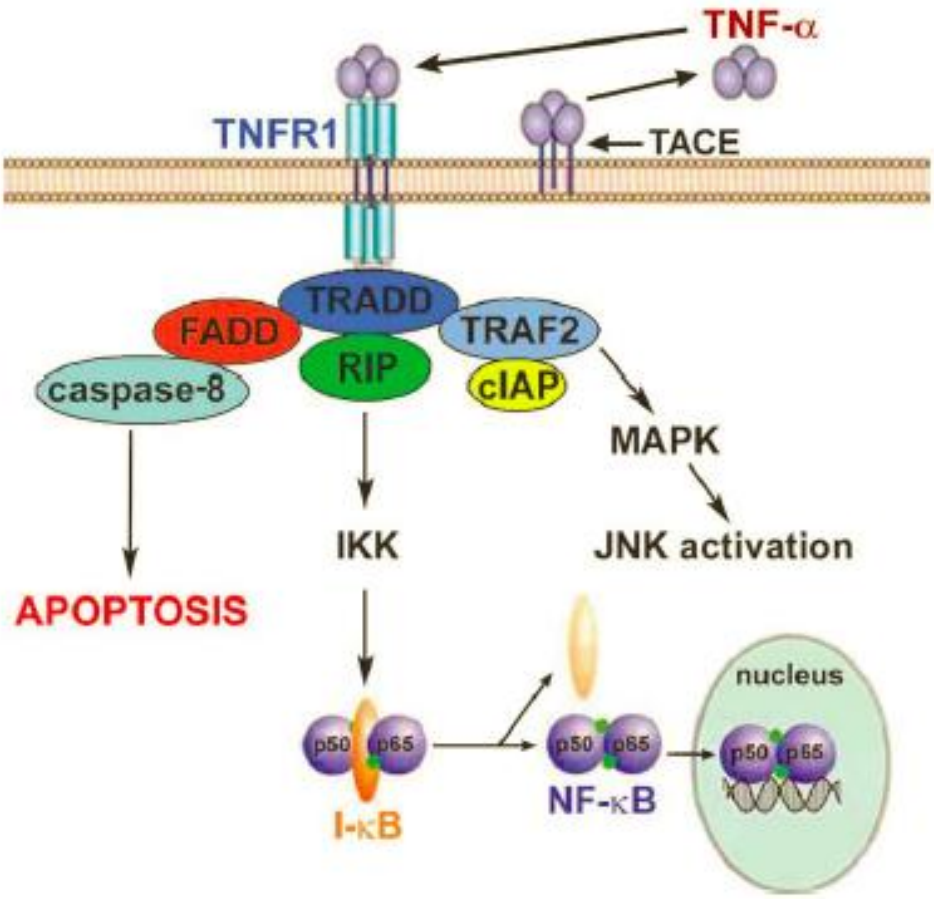


Figure 2-3 Overview of the TNFR1 signaling Pathway [39].

2.4 NF-kB signaling pathways

NF-kB (Nuclear factor kappa beta) is a transcription factor which plays a key role in the regulation of many inflammatory and immune responses. NF-kB is found in almost all animal cell types and involved in cellular responses to several stimuli including : activation by cytokine, free radicals, and oxidative stress. NF-kB can be activated by pro-inflammatory cytokines such as the tumor necrosis factor (TNF-α) or interleukin-1 (IL-1). NF-kB in the brain is a heterodimer consisting of p50 and p65 subunits. Generally in an inactivated state, NF-kappa B is interacting with inhibitor protein, an inhibitor of nuclear factor kappa B (IkB), in the cytoplasm. When cells are stimulated, mainly by pro-inflammatory cytokines, then I kappa B kinase (IKK) is rapidly activated and phosphorylated two critical serines in the N-terminal regulatory domain of IkB. The phosphorylated IkB will be subsequently degraded by the ubiquitin-proteasome pathway. This results in free NF-kB in the cytoplasm and translocates into the nucleus to activate transcription of the target gene [42]. The activation or inactivation of NF-kB is regarded as a key factor regulating the cellular pro-inflammatory and anti-inflammatory balance [43].

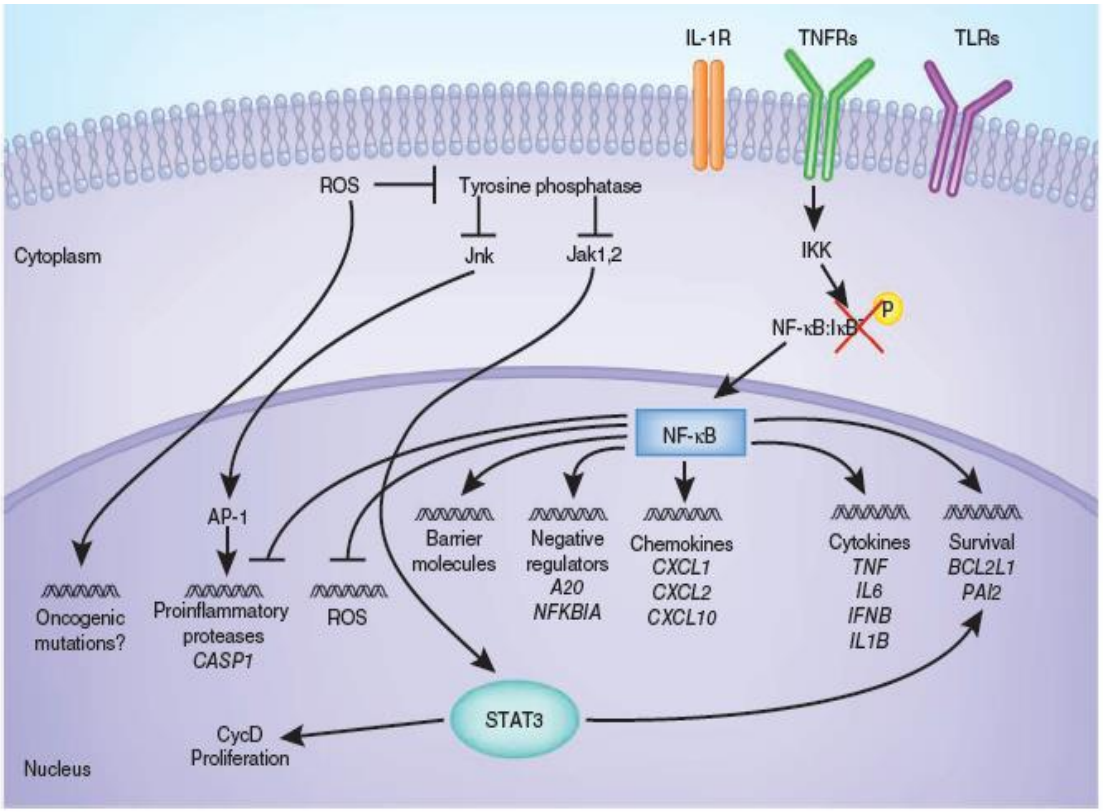


Figure 2-4 NF-kB signaling Pathway [42]

CHAPTER III

MATERIALS AND METHODS

3.1 Animals

Adult male Wistar rats weighing 250-300 grams (National Laboratory Animal Center of Salaya Campus, Mahidol University, Thailand) will be used in this study. The animals were housed in stainless- steel bottom cages and maintained on normal rat food and tap water ad libitum under controlled environmental conditions. All animals received proper care and were used in accordance with the Animal Ethical Committee of the Faculty of Medicine, Chulalongkorn University, Thailand (21/54).

3.2 Study design

The experiment in this study was divided into 4 experiments based on the experimental design as follows :

Experiment I : The study of the effect of acute paracetamol treatment on the CSD induced the changes of pro- inflammatory cytokines

Experiment I : The study of the effect of 5 days paracetamol treatment on the CSD induced the changes of pro- inflammatory cytokines

Experiment II : The study of the effect of 15 days paracetamol treatment on the CSD induced the changes of pro- inflammatory cytokines

Experiment III : The study of the effect of 30 days paracetamol treatment on the CSD induced the changes of pro- inflammatory cytokines

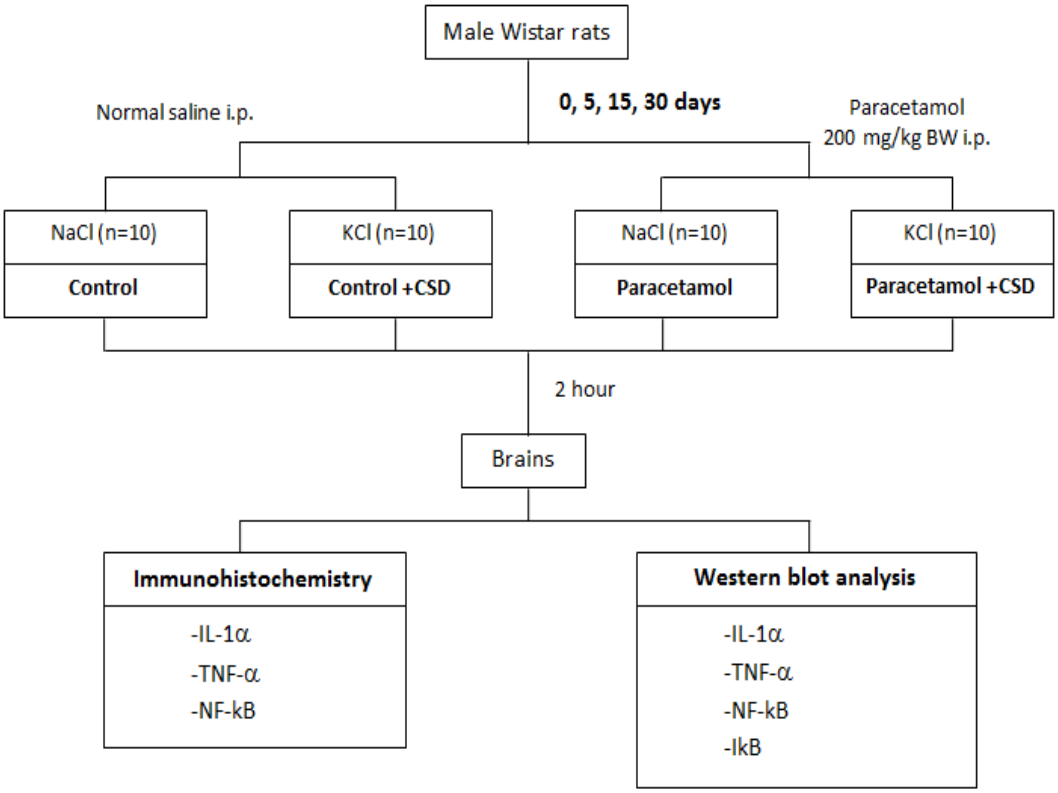


Figure 3-1 Study design of this experiment

Experiment I : The study of the effect of acute paracetamol treatment on the CSD induced the changes of pro- inflammatory cytokines

In this experiment, the rats were divided into two groups as follows: control and paracetamol treated group (APAP). Paracetamol (200 mg/kg of body weight) were injected intraperitonealy for the paracetamol treated group while a normal saline at the same volume were injected to the control group. After 30 min of the last injection, rats will be prepared for CSD induction.

In both experimental groups, the rats were subdivided into 2 subgroups (with and without CSD activation). Rats were anaesthetized by intraperitonealy administration of 60 mg/kg of sodium pentobarbital. An additional dose were given to maintain surgical anesthesia based on the testing of the corneal reflex and response to tail pinch. The CSD were induced based on the same method as previous study [10-11]. The 3 mg of solid KCl were topical applied onto the surface of parietal cortex of the rats with CSD activation while 3 mg solid NaCl were used in the non-CSD induction groups.

Experiment II : The study of the effect of 5 days paracetamol treatment on the CSD induced the changes of pro- inflammatory cytokines

In this experiment, the rats were divided into two groups as follows: control and paracetamol treated group (APAP). Paracetamol (200 mg/kg of body weight) were injected intraperitonealy daily for 5 days to the paracetamol treated group while a normal saline at the same volume were injected daily for 5 days to the control group. After 24 hours of the last injection, rats will be prepared for CSD induction.

In both experimental groups, the rats were subdivided into 2 subgroups (with and without CSD activation) with the same protocol use in experimental I.

Experiment III : The study of the effect of 15 days paracetamol treatment on the CSD induced the changes of pro- inflammatory cytokines

In this experiment, the rats were divided into two groups as follows: control and paracetamol treated group (APAP). The rats in this experiment were treated with the same protocol used in experimental II **except** the duration of paracetamol and normal saline treatment were 15 days.

Experiment IV : The study of the effect of 30 days paracetamol treatment on the CSD induced the changes of pro- inflammatory cytokines

In this experiment, the rats were divided into two groups as follows: control and paracetamol treated group. The rats in this experiment were treated with the same protocol used in experimental II **except** the duration of paracetamol and normal saline treatment were 30 days.

3.3 Methods

3.3.1 Surgical operation and induction of cortical spreading depression (CSD)

The rats were anaesthetized by intraperitoneal injection with sodium pentobarbital (dose 60 mg/kg body weight). The rats were fixed to the head holder on the surgical frame and tracheotomy was done. Craniotomy was performed on rats in 2 mm diameter on the parietal bone at 7 mm posterior and 1 mm lateral to the bregma. The dura were opened by using microneedle. The CSD was induced by the topical application of 3 mg of solid KCl onto the surface of parietal cortex of the rats brain.

3.3.2 Sample collection

3.3.2.1 Perfusion and tissue preparation

After two hours of KCl or NaCl application, rats were humanely killed by injection of an excessive dose of sodium pentobarbital. Blood sample were collected and separated serum for liver functions test .Then, perfused transcardially with 250 mL of phosphate buffer and followed by 250 mL of 4% paraformadehyde in 0.1M PBS pH 7.4. The liver and brain was rapidly removed. The liver were cut at cross sections of 5 mm thickness (3 slices per liver). The thick brain slices were cut at 3 mm anterior to bregma and then immersed in a 4% paraformadehyde 0.1M phosphate buffer. After overnight fixation, tissues were processed for paraffin embedding. These sample were processed for the hematoxylin-eosin staining and immunohistochemistry.

3.3.2.2 Preparation of Fresh frozen tissue

After two hours of KCl or NaCl application, rats were humanely killed by injection of an excessive dose of sodium pentobarbital. Then, the brain was rapidly removed. The frontal cortex ipsilateral was collected and was immediately transferred into the liquid nitrogen. All samples were kept at -80 ºc until analyzed. These sample were processed for the western blotting study.

3.4 Liver function test

3.4.1 Liver enzyme levels & Histology analysis

In order to monitor the effect of paracetamol on the hepatotoxicity, in this experiments, the three liver enzyme (alanine aminotransferase ; AST, aspartate aminotransferase ; ALT and alkaline phosphatase ; ALP) was measured in all animal. Hematoxylin-eosion staining was used to evaluate the extent of paracetamol on the hepatotoxicity. We observed on change in hepatocyte morphology or evidence of inflammation. Liver from rats were examined for all experiment groups.

3.5 Immunohistochemical study

The serial coronal brain sections was cut at 3 μm thickness by using a microtome, collected in 1 in 12 series. Total 4 sections were collected from each animal. The collected sections were placed on the super-frost plus glass slide and heated at 60°C overnight before processing for deparaffinization.

Brain sections (3 μm thick) were deparaffinised and incubated in citrate buffer (pH 6.6) at 95°c to retrieve the antigenicity. Endogenous peroxidase blocking by incubated with 3% hydrogen peroxide in 50% methanol. After that, the sections were processed for blocked non-specific binding by incubated with 5% normal horses serum and incubated with primary antibody for identification of the expression of pro-inflammation cytokines and transcription factor NF-kB in the cerebral cortex. For immunohistochemistry, rabbit anti-IL-1α (sc-7929; Santa Cruz Biotechnology, USA), anti-TNF-α (sc-1350; Santa Cruz Biotechnology, USA) and anti-p65 NF-kB antibodies (sc-109; Santa Cruz Biotechnology, USA) were used at 1:100 dilution. After incubation with primary antibody, the sections were incubated for 30 min with anti-rabbit for IL-1α and NF-kB labeling and incubated with anti-goat for TNF-α labeling (Dako EnVision kits, Glostrup, Denmark). The product of immunohistochemical reaction was visualized using the DAB reagent and counterstained with hematoxylin (Dako, Glostrup, Denmark). Sections were dehydrated in a graded series of ethanol, mounted, cover-slipped with Permount, and examined with a light microscope.

To determine the expression of pro-inflammation cytokines in the sample, 4 brain sections were selected from each animal. The number of IL-1α and TNF-α immunoreactive cells were counted in the area of lamina I-IV of the cerebral cortex ipsilateral to CSD activation. The data were reported as the % of average number of positive cells per 2.5 square millimeters (cells/ 2.5 mm^2). The identity of each rat in the treatment and the control groups was blinded during the count process.

3.6 Western blot analysis

Protein extraction

Frontal cortex brain sample were washed twice in 10 ml ice-cold PBS (5mM Tris-HCl, pH 7.4 autoclave) and homogenized at 4ºc in RIPA buffer (Cell Signaling, USA) containing a protease and phosphatase inhibitor cocktails (Thermo, USA). Then, the lysate was sonicated three times for 5 sec, and incubated on ice for 45 min. The samples were centrifuged at 12,000 x g for 15 min at 4ºc. The supernatants were collected as protein extracts and stored at -80°c until use. The concentration of protein was quantified by using the BCA protein assay kits (Thermo, USA) with bovine serum albumin as standard.

Western blotting

A 40 μg of the whole protein extracts were mixed with 4x SDS protein sample buffer (240 mM Tris-HCl (pH 6.8), 40% Glycerol, 8% SDS, 0.04% Bromophenol blue, 5% β-mercaptoethanol) and boiled at 95ºc for 5 min. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 volts for 120 min and transferred into nitrocellulose membranes at 0.35 Amp constant for 1.30 hr using the Mini Trans-Blot® Electrophoresis Transfer Cell (BioRad, USA). A non-specific protein binding was blocked by incubation the membranes with TBST containing 5% BSA for 1 hr at room temperature. The blot was probed with the primary antibody: IL-1α (dilution 1:100) (sc-7929; Santa Cruz Biotechnology, USA), TNF-α (dilution 1:100) (sc-1350; Santa Cruz Biotechnology, USA), NF-kB p65 (dilution 1: 1000) (#3034; Cell signaling, USA), phospho-NF-kB p65 (dilution 1:1000) (#3031; Cell signaling, USA) and phospho-IkB (dilution 1:1000) (#9246; Cell signaling, USA) β-actin (dilution 1:3000) (AC-15 ; Sigma, USA) in 5% BSA in TBST at 4ºc overnight. Subsequently, the blot was washed three times for 10 min in 0.1% Tween 20 in TBS before incubation with a horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature. The blot was washed three times for 10 min in 0.1% Tween 20 in TBS. The immunoreactive bands were visualized by using the SuperSignal® West Pico Chemiluminescence Substrate Kits (GE Healthcare Life Science, USA). The band intensity were semi-quantified by

using Image J software (Scion crop; Frederick, MD) and results are expressed as protein/β-actin ratio in the same samples.

3.7 Data Analysis

All data were presented as mean and standard deviation (SD). All statistical analyses were performed using the GraphPad Prism software version 6 (GraphPad, San Diego, CA, USA). Statistically difference between groups were tested by one-way analysis of variance (one-way ANOVA), followed by Bonferroni post hoc test. Differences were considered statistically significant at $p < .05$.

CHAPTER IV

RESULTS

4.1 The effect of chronic paracetamol treatment on the hepatotoxicity

In order to clarify whether the paracetamol treatment in all experimental groups induces the hepatotoxicity, or not the level of main three important liver enzymes (AST, ALT, and ALP) were measured. The results demonstrated that intraperitoneal administration of paracetamol at the doses of 200 mg/kg BW for a period of 0, 5, 15, and 30 days had no effect on the levels of AST, ALT, and ALP. Compared to the control group, there was no significant difference in the levels of all liver enzymes measured in this study. The data are shown in table 4-1.In addition, the histological examination of the liver was normal. No change in hepatocyte morphology or evidence of inflammation was observed.

These data indicate that acute and chronic paracetamol treatment (dose 200 mg/kg BW) had no effect on the liver function.

Table 4-1 The effect of chronic paracetamol treatment at four different time points (0, 5, 15, and 30 days) on the alanine aminotransferase (AST), aspartate aminotransferase (ALT), and alkaline phosphatase (ALP) levels in rats serum.

Liver enzymes levels (μ/L)	APAP administration (days)	Groups			
		Control (n=5)	Control+CSD (n=5)	APAP (n=5)	APAP+CSD (n=5)
AST (45.7 - 80.8)	0	70.01±10.53	65.05±13.04	71.09±9.98	64.77±12.39
	5	65.43±12.11	66.14±12.02	68.91±11.07	69.87±13.99
	15	72.09±11.21	73.16±9.89	59.98±14.88	61.99±11.78
	30	61.38±11.54	77.60±7.12	60.50±13.26	59.86±16.71
ALT (35-80)	0	72.01±11.35	69.09±15..97	70.78±11.78	71.05±13.45
	5	64.76±15.04	55.14±11.23	67.88±14.05	65.91±15.98
	15	58.45±14.23	49.98±11.54	56.45±12.71	51.34±15.34
	30	71.00±11.05	54.40±17.91	57.09±11.09	49.40±10.41
ALP (56.8-128)	0	91.45±14.13	94.18±15.67	88.89±14.23	93.71±25.41
	5	85.57±15.09	95.01±14.21	92.07±9.98	89.04±15.67
	15	81.21±13.24	82.65±17.54	86.62±10.25	78.89±13.45
	30	89.63±19.09	89.00±11.00	73.83±21.04	71.05±14.51

The data are expressed as the mean ± SD.

4.2 The effect of chronic paracetamol treatment on the CSD-induced the expression of interlukin-1 alpha (IL-1α) in the cerebral cortex

The results from western blot analysis demonstrated that, the CSD activation without paracetamol treatment could not induce the expression of IL-1α in the cerebral cortex. The paracetamol treatment for 0 and 5 days with and without the combination of CSD activation also had no effect on the expression of IL-1α. However, the chronic treatment with paracetamol without CSD for 15 and 30 days could significantly increase the IL-1α expression as compared with the control and control groups with CSD activation. In addition, the paracetamol treatment for 15 and 30 days in combination with CSD activation significantly increased the expression of IL-1α as compared with those of the control group. The data are shown in Figure 4-1, 4-3, and 4-5.

Interestingly, our results have found that when comparing the level of IL-1α expression between the paracetamol treated group and the paracetamol treated group with CSD activation, the significant difference in the level of IL-1α expression was only demonstrated among the groups with 15 days treatment of paracetamol. No significant difference was observed between the groups with 30 days treatment of paracetamol.

The study of IL-1α expression in the cerebral cortex by the immunohistochemical technique demonstrated overall results with a similar pattern as those of the western blot analysis, as the data demonstrated in Figure 4-2, 4-4, and 4-6. The number of positive cells are demonstrated in Table 4-2. For the animals with 0 and 5 days paracetamol treatment, neither paracetamol treatment with and without CSD activation could induce the IL-1α expression in the cerebral cortex. Parallel with the result from western blot analysis, the significant increase in the number of IL-1α immune-reactive cells was observed in the cerebral cortex obtained from the paracetamol treatment group with and without CSD activation for 15 and 30 days.

The results obtained from this part of the study have indicated that short term paracetamol treatment (0 and 5 days) had no effect on the expression of IL-1α. However, the long term paracetamol treatment (15 and 30 days) either in combination with or without CSD activation could induce an increase in the expression of IL-1α in the cerebral cortex.

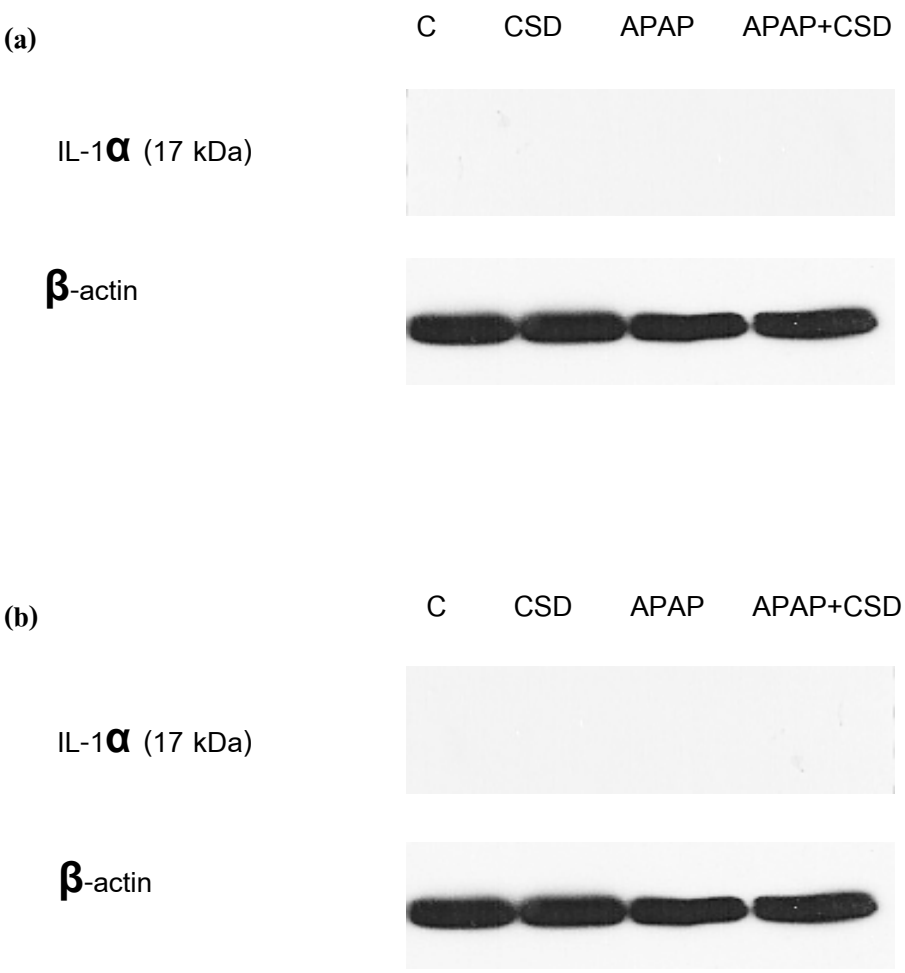
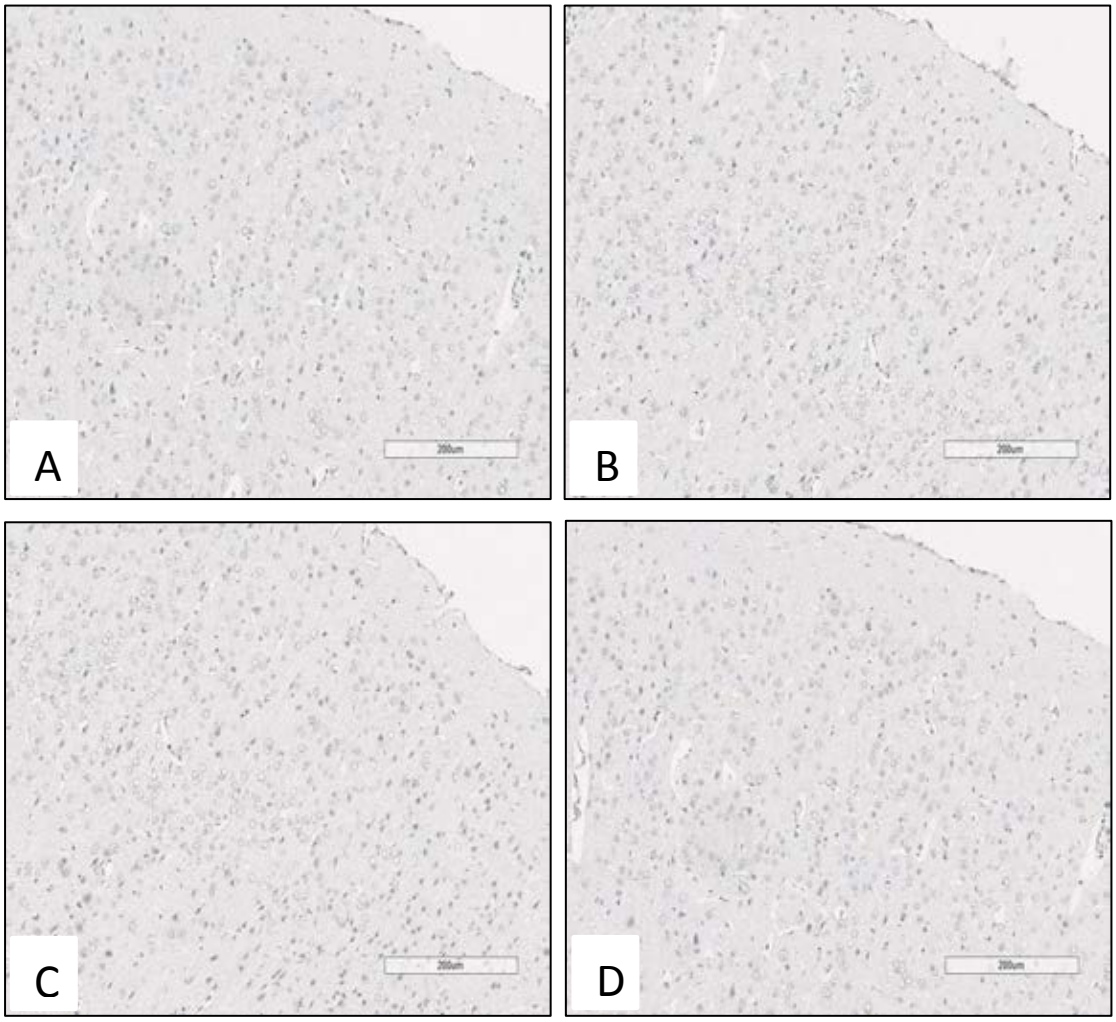


Figure 4-1 The effect of (a) 0 days and (b) 5 days paracetamol treatment on CSD-induced an expression of IL-1α in the cerebral cortex detected by western blotting.

Abbreviation : C = Control, CSD = Control with CSD activation group, APAP = Paracetamol group, APAP+CSD = Paracetamol with CSD activation group.

(a)



(b)

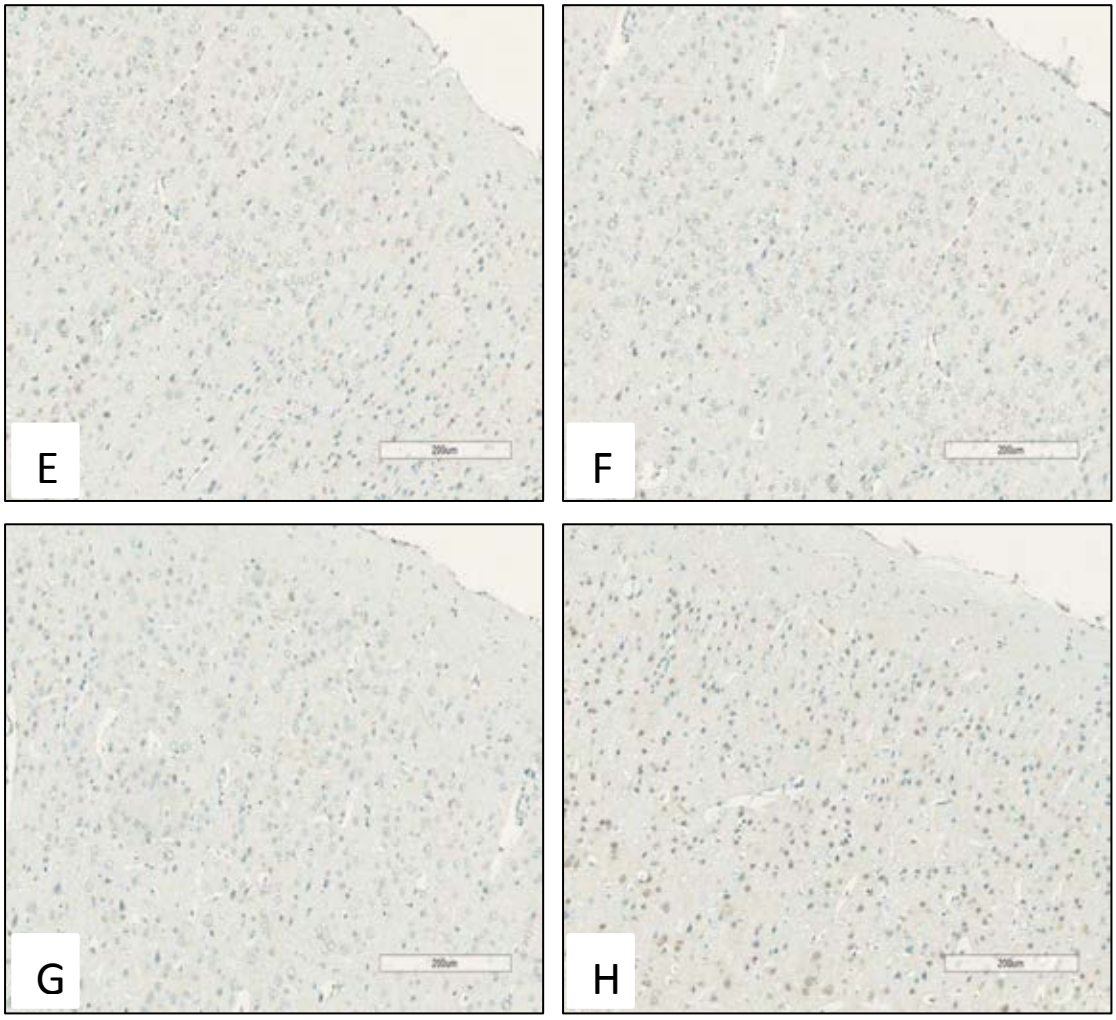


Figure 4-2 The effect of (a) 0 days and (b) 5 days paracetamol treatment on the IL-1α expression in the brain. The photomicrographs showing the expression of the IL-1α immunoreactive cells in the cerebral cortex obtained from the control (A, E), control with CSD activation (B, F), paracetamol (C, G), and paracetamol with CSD activation group (D, H).

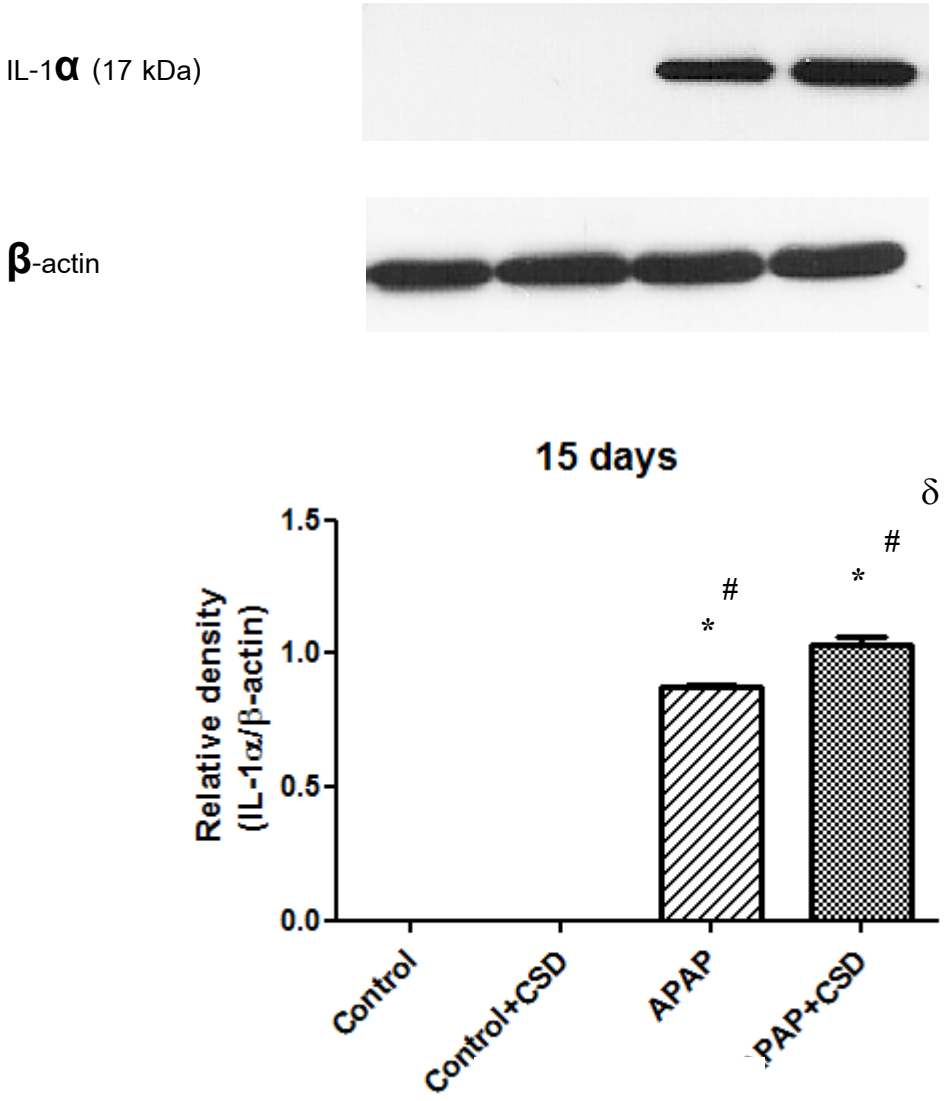


Figure 4-3 The effect of 15 days paracetamol treatment on CSD-induced an expression of IL-1α in the cerebral cortex. Protein levels were analyzed by western blotting. Quantitative data are expressed as relative density of IL-1α to β-actin. The values represent the mean ± SD (n=4). $^{*}p<0.05$ compared with the control group. $^{\#}p<0.05$ compared with control with CSD activation group. $^{\delta}p<0.05$ compared with paracetamol group.

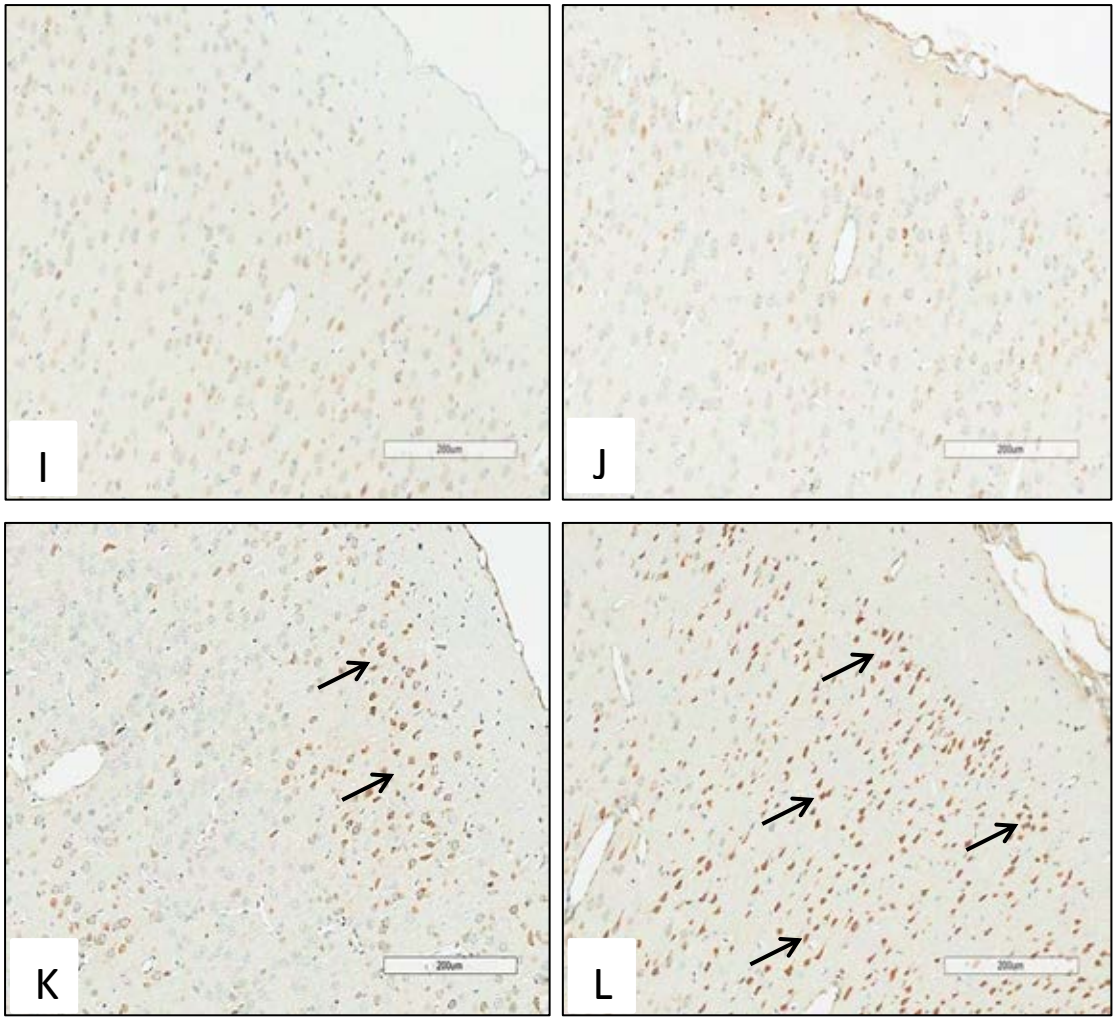


Figure 4-4 The photomicrograph shows localization of the IL-1α immunoreactive cells (arrow) in the cerebral cortex at 15 days obtained from the control (I), control with CSD activation (J), paracetamol (K), and paracetamol with CSD activation group (L).

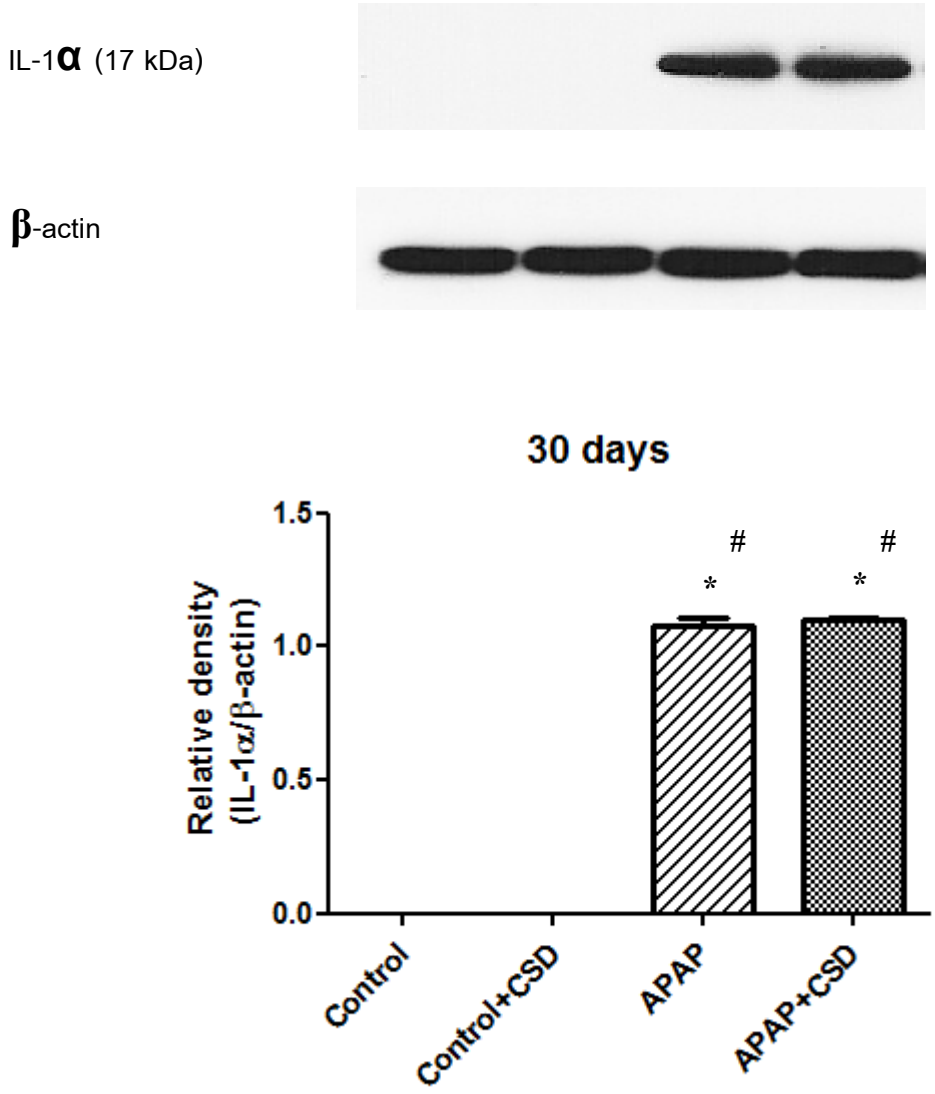


Figure 4-5 The effect of 30 days paracetamol treatment on CSD-induced an expression of IL-1α in the cerebral cortex. Protein levels were analyzed by western blotting. Quantitative data are expressed as relative density of IL-1α to β-actin. The values represent the mean ± SD (n=4). $^{*}p<0.05$ compared with the control group. $^{\#}p<0.05$ compared with control with CSD activation group.

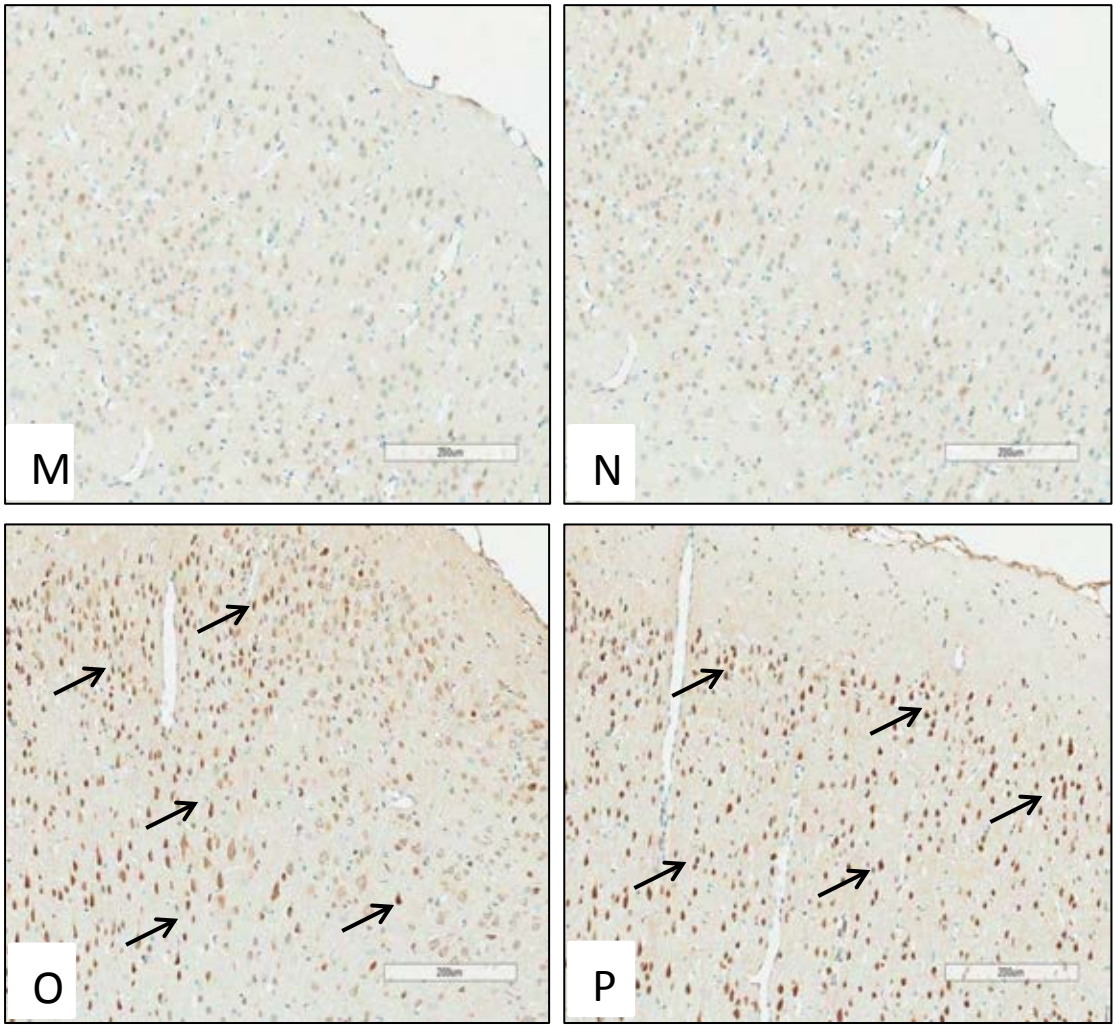


Figure 4-6 The photomicrograph shows localization of the IL-1α immunoreactive cells (arrow) in the cerebral cortex at 30 days obtained from the control (M), control with CSD activation (N), paracetamol (O), and paracetamol with CSD activation group (P).

Table 4-2 Effect of paracetamol treatment for four different time points on the pro-inflammatory cytokine IL-1α expression in the rat's cerebral cortex.

Paracetamol administration (days)	Control (n=5)	Control+CSD (n=5)	Paracetamol (n=5)	Paracetamol+CSD (n=5)
0	0	0	0	0
5	0	0	0	0
15	5.03±6.01	4.11±4.17	35.46±9.81$^{*,\#}$	61.34±11.32$^{*,\#,\delta}$
30	4.07±4.13	3.19±5.26	64.89±11.47$^{*,\#}$	69.48±10.24$^{*,\#}$

The data are expressed as the mean ± SD.

$^{*}p<0.05$ compared with the control group in the same time point.

$^{\#}p<0.05$ compared with the control with CSD activation group in the same time point.

$^{\delta}p<0.05$ compared with the paracetamol group in the same time point.

4.3 The effect of chronic paracetamol treatment on the CSD-induced the expression of tumor necrosis factor (TNF-α) in the cerebral cortex

Based on the experiment in which measurements were done through western blot analysis, results showed that the induction of CSD by KCl application did not induce the expression of TNF-α in the cerebral cortex. In addition, the expression of TNF-α was not affected by the short term paracetamol treatment (0 and 5 days) independent of including or not including CSD activation. In contrast to this, the TNF-α expression was affected by long term paracetamol treatment for 15 and 30 days in as compared with the control group. Further, expression of TNF-α had significant increased compared with the control and the control with CSD activation group when long term paracetamol treatment (15 and 30 days) in combination with CSD activation was administered. From the perspective of comparing the effect of long term paracetamol treatment between with and without CSD activation, only a significant difference in the level of TNF-α expression was observed in the 15 days paracetamol treatment. At the treatment of 30 days with paracetamol, no significant difference was observed when comparing the expression of TNF alpha between the animal with and without CSD activation. The data are shown in Figure 4-7, 4-9, and 4-10.

Moreover the results of the study of TNF-α expression in the cerebral cortex by using the immunohistochemical technique demonstrated similar results with those from the western blot analysis. Data are shown in Figure 4-8, 4-11, and 4-12. In both cased, short term paracetamol treatmentin the condition both with and without CSD activation, TNF-α expression in the cerebral cortex was not induced. In a similar context as in western blot analysis, the number of TNF-α immunoreactive cells in the cerebral cortex obtained from paracetamol treatment for 15 and 30 days in combination with and without CSD activation had significantly increased. The data are shown in Table 4-3.

As the results from this study, the short term paracetamol treatment (0 and 5 days) had no significant effect on the expression of TNF-α. In contrast, long term paracetamol treatment (15 and 30 days) both with and without CSD activation induced a significant increase in the expression of TNF-α in the cerebral cortex.

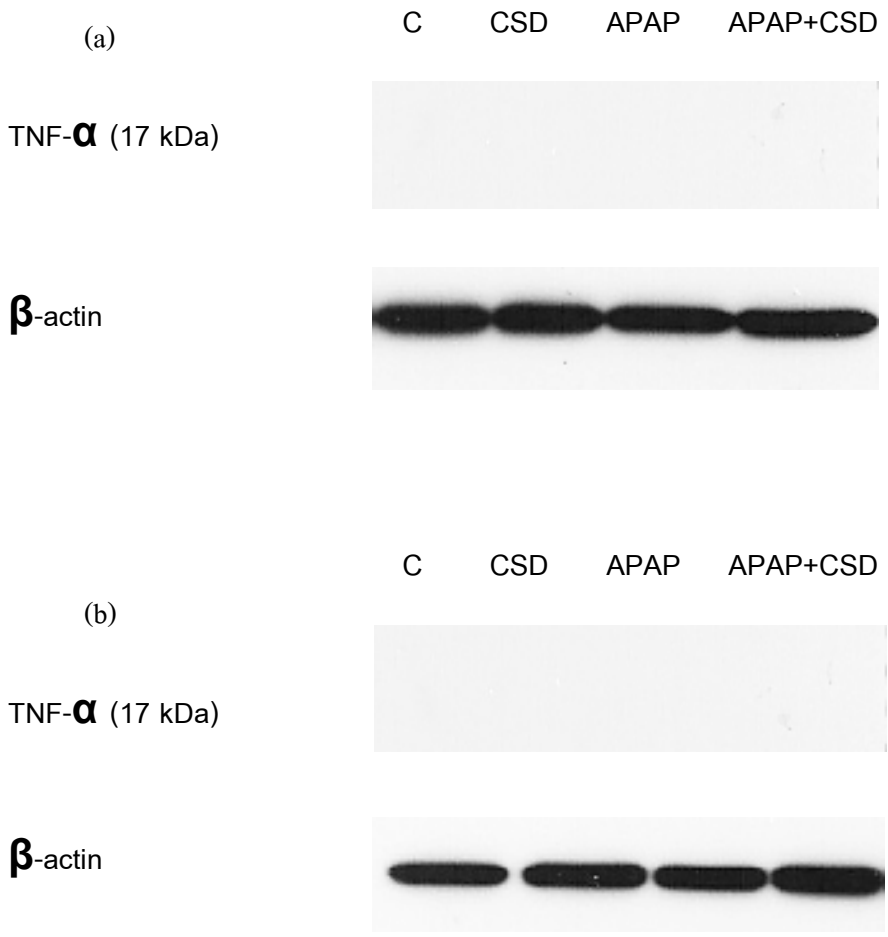
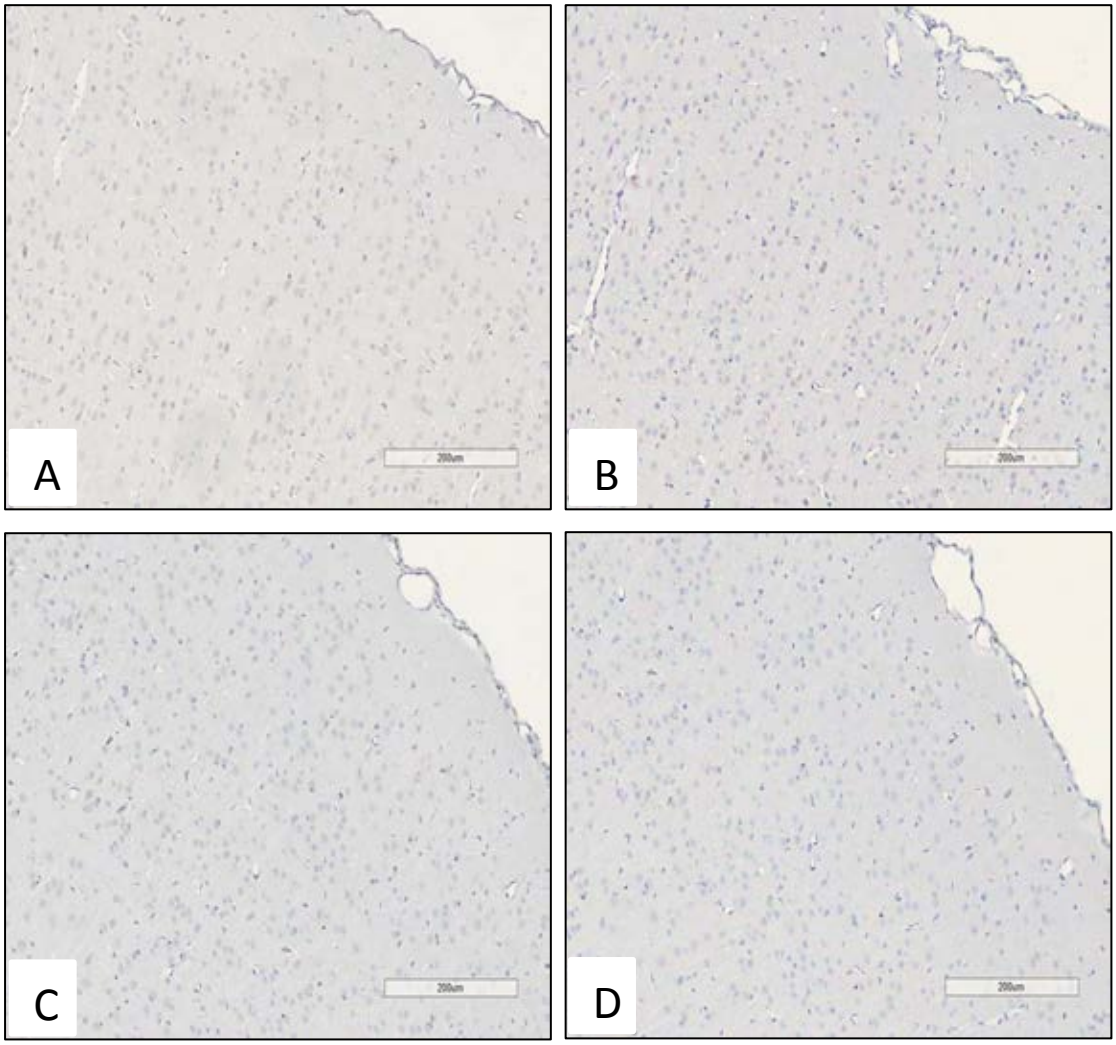


Figure 4-7 The effect of (a) 0 days and (b) 5 days paracetamol treatment on CSD-induced an expression of TNF-α in the cerebral cortex detected by western blotting.

Abbreviation : C = Control, CSD = Control with CSD activation group, APAP = Paracetamol group, APAP+CSD = Paracetamol with CSD activation group.

(a)



(b)

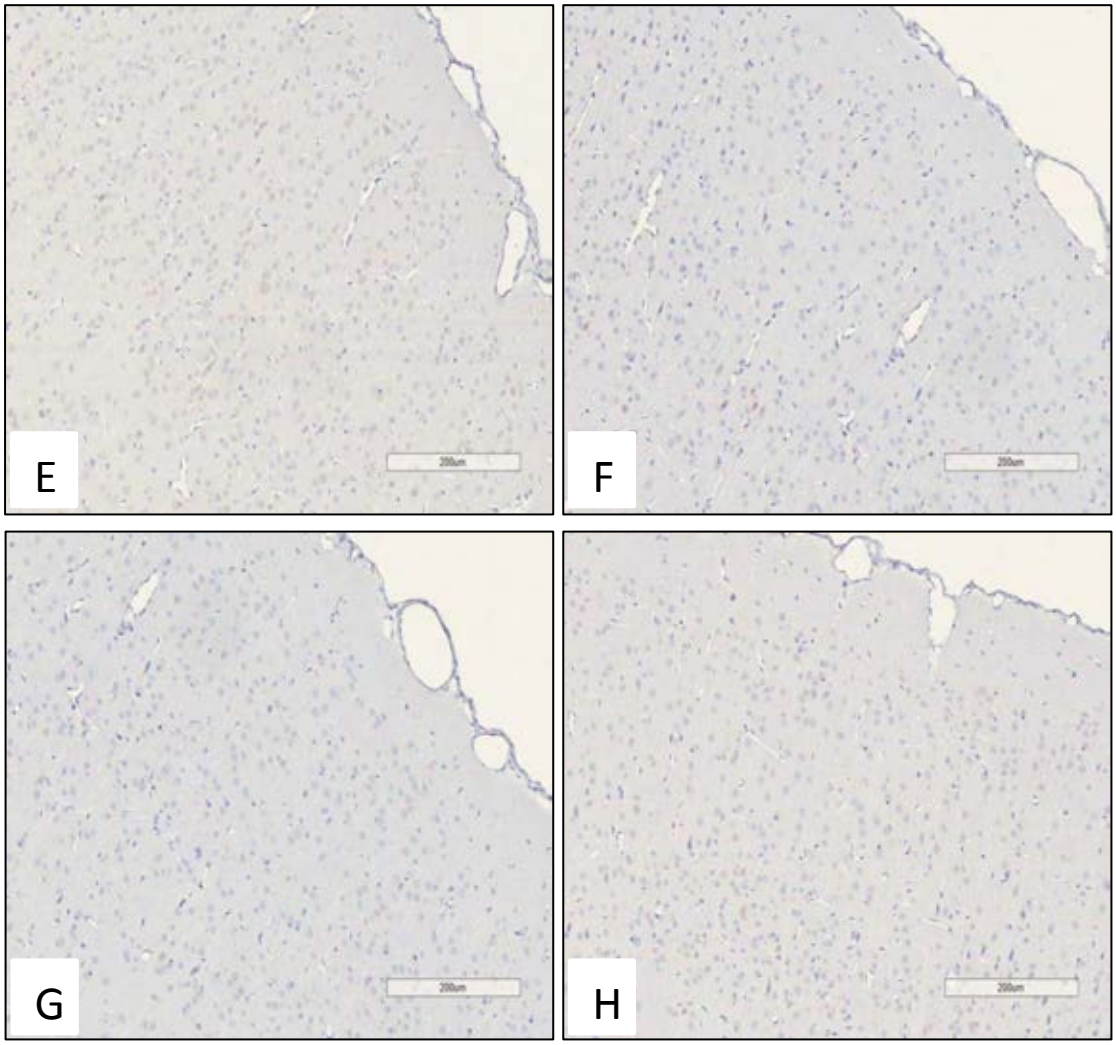


Figure 4-8 The effect of (a) 0 days and (b) 5 days paracetamol treatment on the TNF-α expression in the brain. The photomicrographs showing the expression of the TNF-α immunoreactive cells in the cerebral cortex obtained from the control (A, E), control with CSD activation (B, F), paracetamol (C, G), and paracetamol with CSD activation group (D, H).

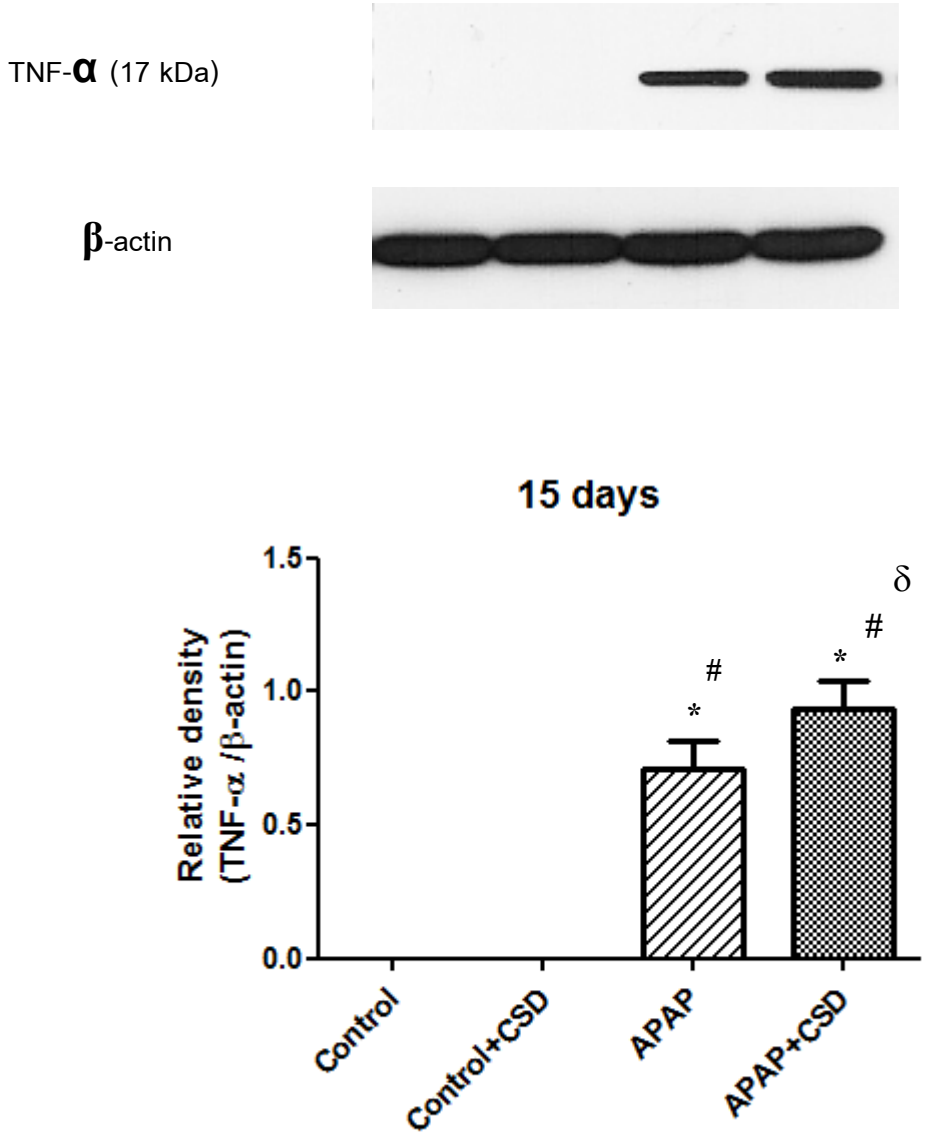


Figure 4-9 The effect of 15 days paracetamol treatment on CSD-induced an expression of TNF-α in the cerebral cortex. Protein levels were analyzed by western blotting. Quantitative data are expressed as relative density of TNF-α to β-actin. The values represent the mean ± SD (n=4). $^{*}p<0.05$ compared with the control group. $^{\#}p<0.05$ compared with control with CSD activation group. $^{\delta}p<0.05$ compared with paracetamol group.

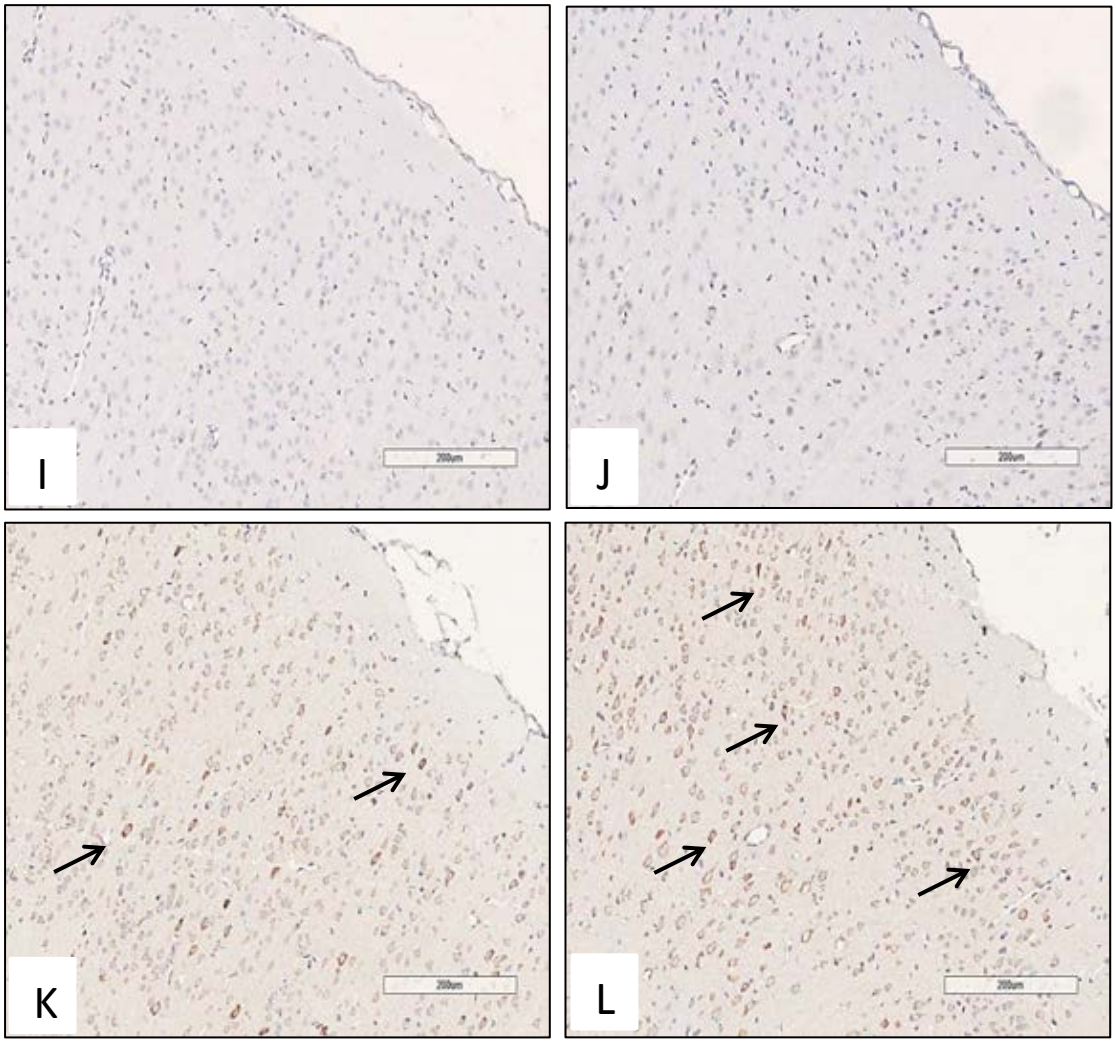


Figure 4-10 The photomicrograph shows localization of the TNF-α immunoreactive cells (arrow) in the cerebral cortex at 15 days obtained from the control (I), control with CSD activation (J), paracetamol (K), and paracetamol with CSD activation group (L).

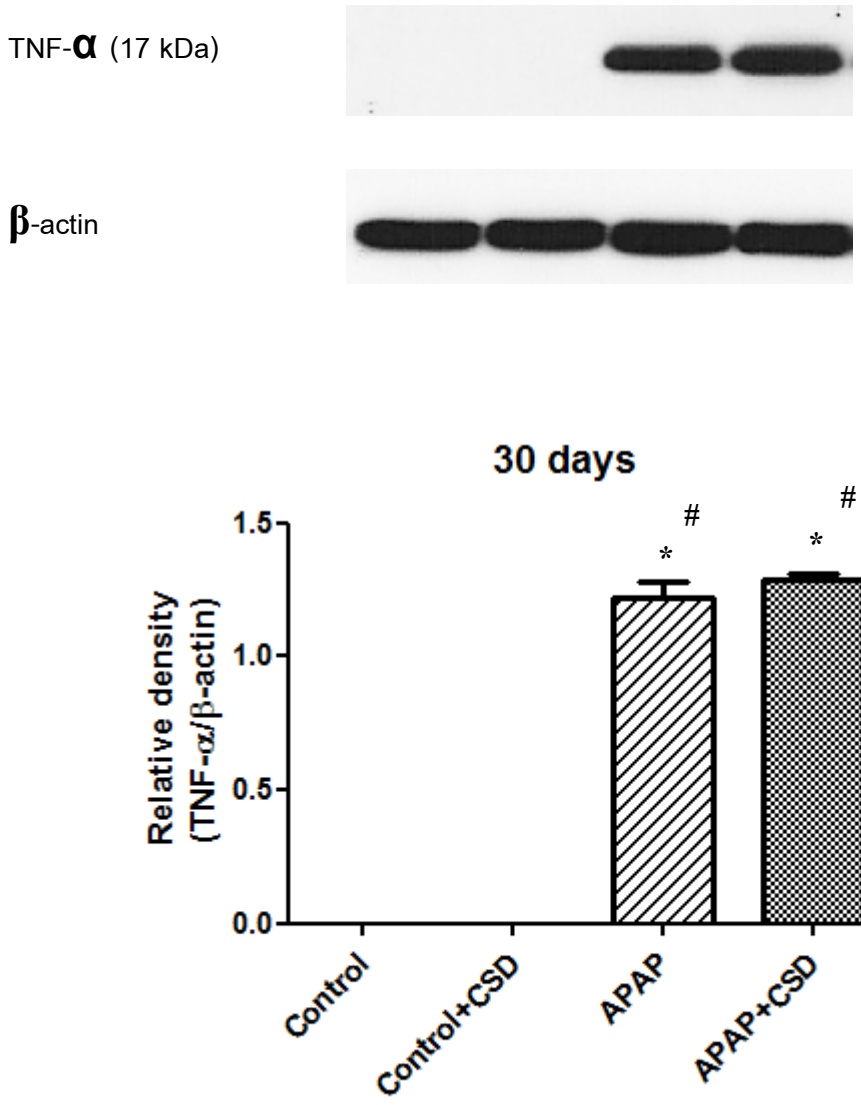


Figure 4-11 The effect of 15 days paracetamol treatment on CSD-induced an expression of TNF-α in the cerebral cortex. Protein levels were analyzed by western blotting. Quantitative data are expressed as relative density of TNF-α to β-actin. The values represent the mean ± SD (n=4). $^{*}p<0.05$ compared with the control group. $^{\#}p<0.05$ compared with control with CSD activation group.

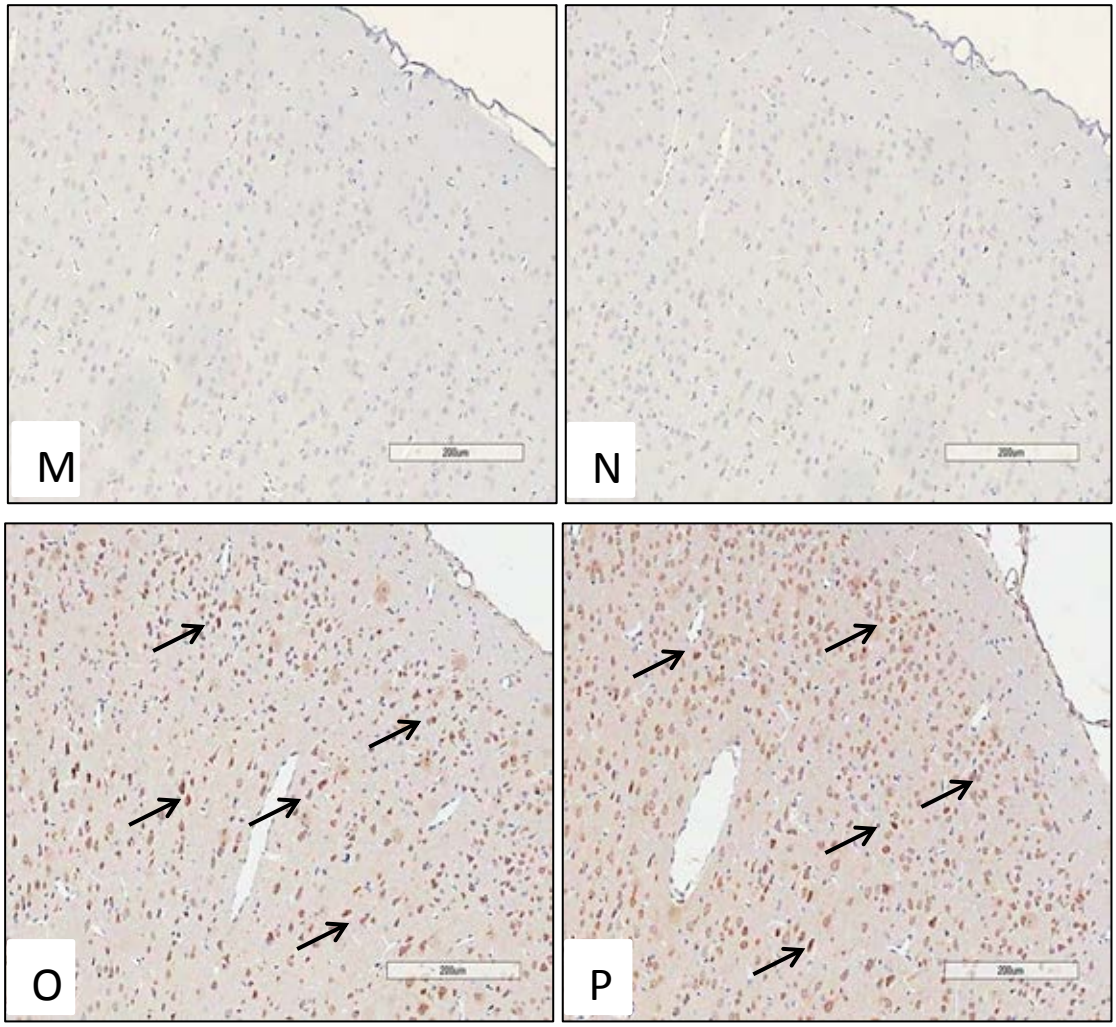


Figure 4-12 The photomicrograph shows localization of the TNF-α immunoreactive cells (arrow) in the cerebral cortex at 30 days obtained from the control (M), control with CSD activation (N), paracetamol (O), and paracetamol with CSD activation group (P).

Table 4-3 Effect of paracetamol treatment for four different time points on the pro-inflammatory cytokine TNF-α expression in the rat's cerebral cortex.

Paracetamol administration (days)	Control (n=5)	Control+CSD (n=5)	Paracetamol (n=5)	Paracetamol+CSD (n=5)
0	0	0	0	0
5	0	0	0	0
15	2.95±4.06	3.37±4.38	40.87±9.75$^{*,\#}$	59.39±10.28$^{*,\#,\delta}$
30	1.67±5.31	3.19±4.12	63.45±13.02$^{*,\#}$	73.37±12.01$^{*,\#}$

The data are expressed as the mean ± SD.

$^{*}p<0.05$ compared with the control group in the same time point.

$^{\#}p<0.05$ compared with the control with CSD activation group in the same time point.

$^{\delta}p<0.05$ compared with the paracetamol group in the same time point.

4.4 The effect of chronic paracetamol treatment on the CSD-induced the activation of the NF-kB signaling pathway

The objective of this study is to investigate the mechanism in which paracetamol induces the increase in pro-inflammatory cytokines. We focused on the activation of the transcription factor NF-kB as a target. We have detected the expression of NF-kB (Total NF-kB), phospho-NF-kB (activated form of NF-kB), and phospho-IkB (phosphorylation form of IkB) at the protein levels, in which 3 parameters are involved in the NF-kB signaling pathway.

4.4.1 Expression of NF-kB

The expression of NF-kB p65 were quantitatively detected by western blotting. There were no significant differences in the expression of NF-kB p65 in all groups as compared with the control group at the same duration. The data are shown in Figure 4-13, 4-15, 4-17, and 4-19.

The expression of NF-kB p65 in the cerebral cortex through imunostaining showed similar result with those obtained from western blot analysis. The data are shown in Figure 4-14, 4-16, 4-18, and 4-20. The number of positive cells are demonstrated in Table 4-4.

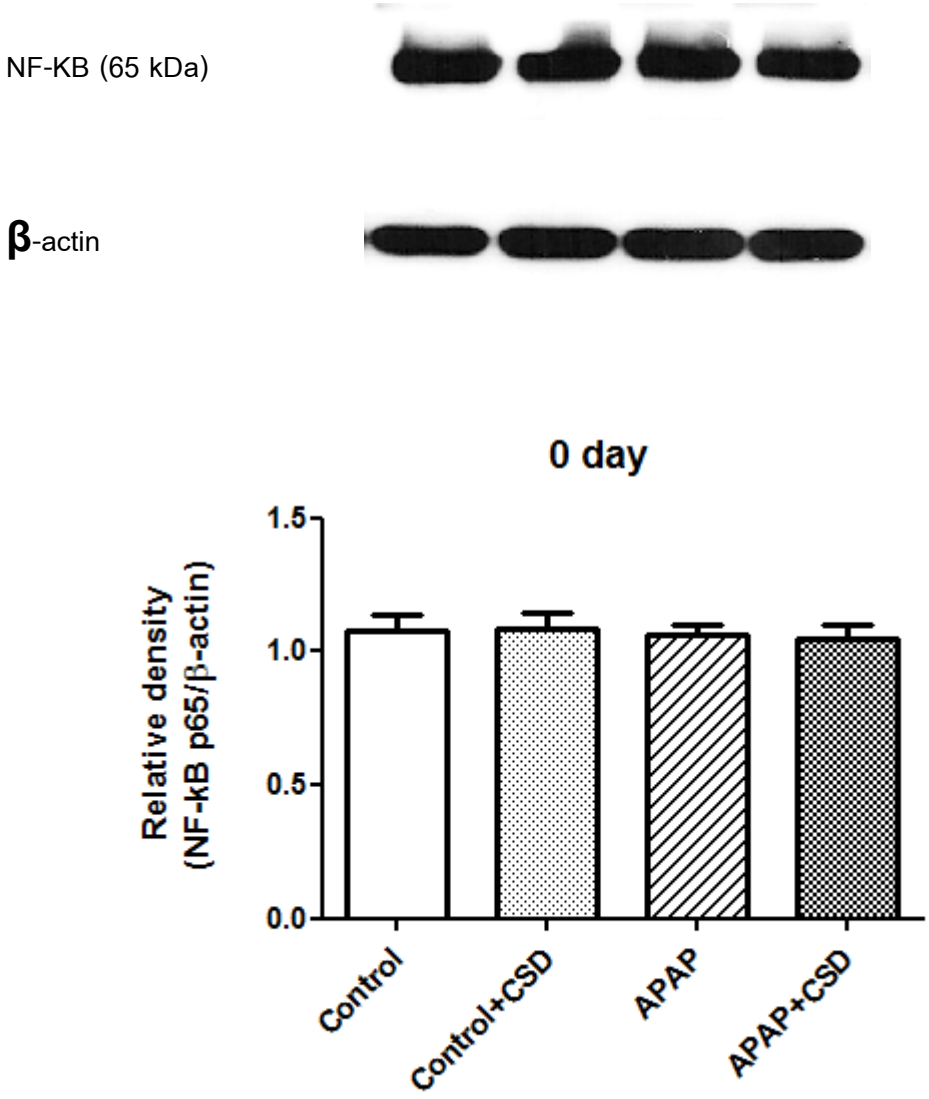


Figure 4-13 The effect of 0 days paracetamol treatment on CSD-induced an expression of the transcription factor NF-kB p65 in the cerebral cortex. Protein levels were analyzed by western blotting. Quantitative data are expressed as relative density to β-actin. The values represent the mean ± SD (n=4).

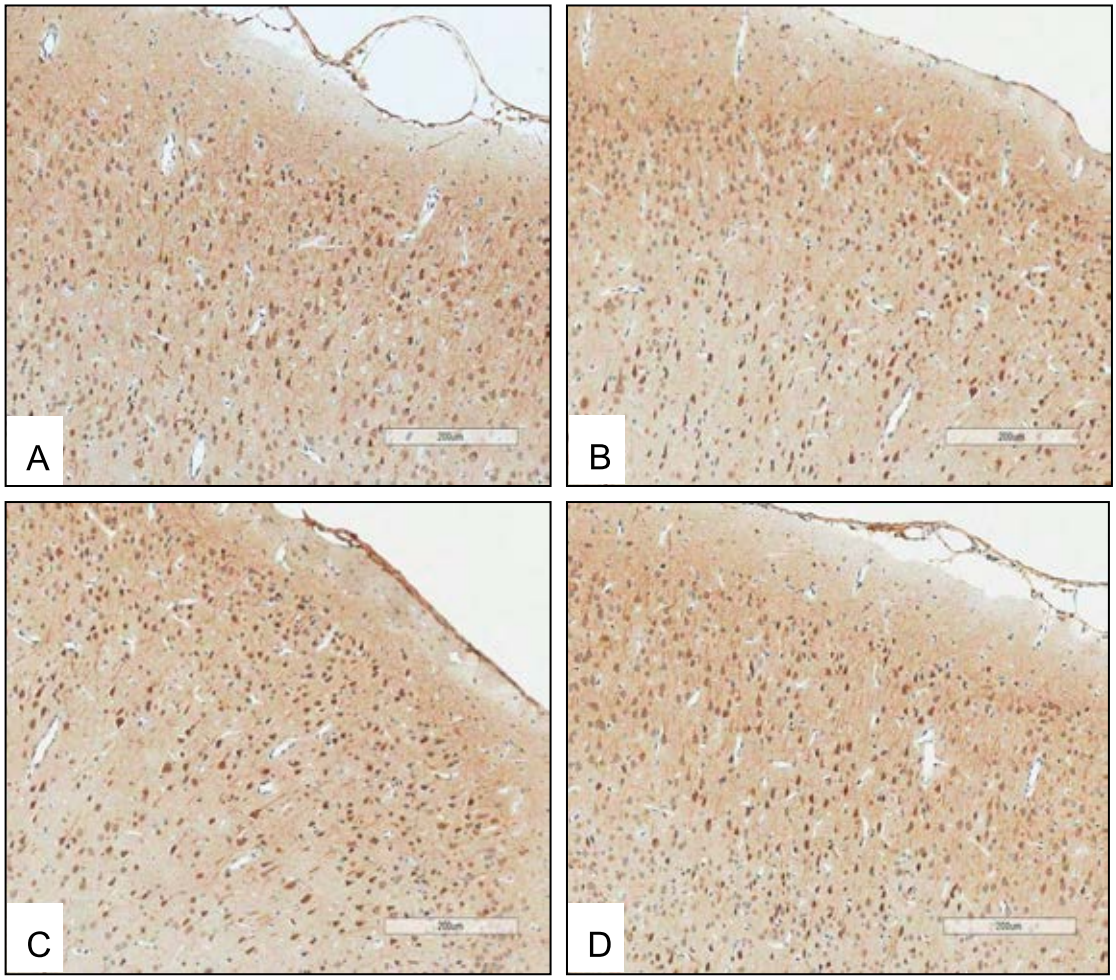


Figure 4-14 The photomicrograph shows localization of the NF-kB immunoreactive cells (dark brown cells) in the cerebral cortex at 0 days obtained from the control (A), control with CSD activation (B), paracetamol (C), and paracetamol with CSD activation group (D).

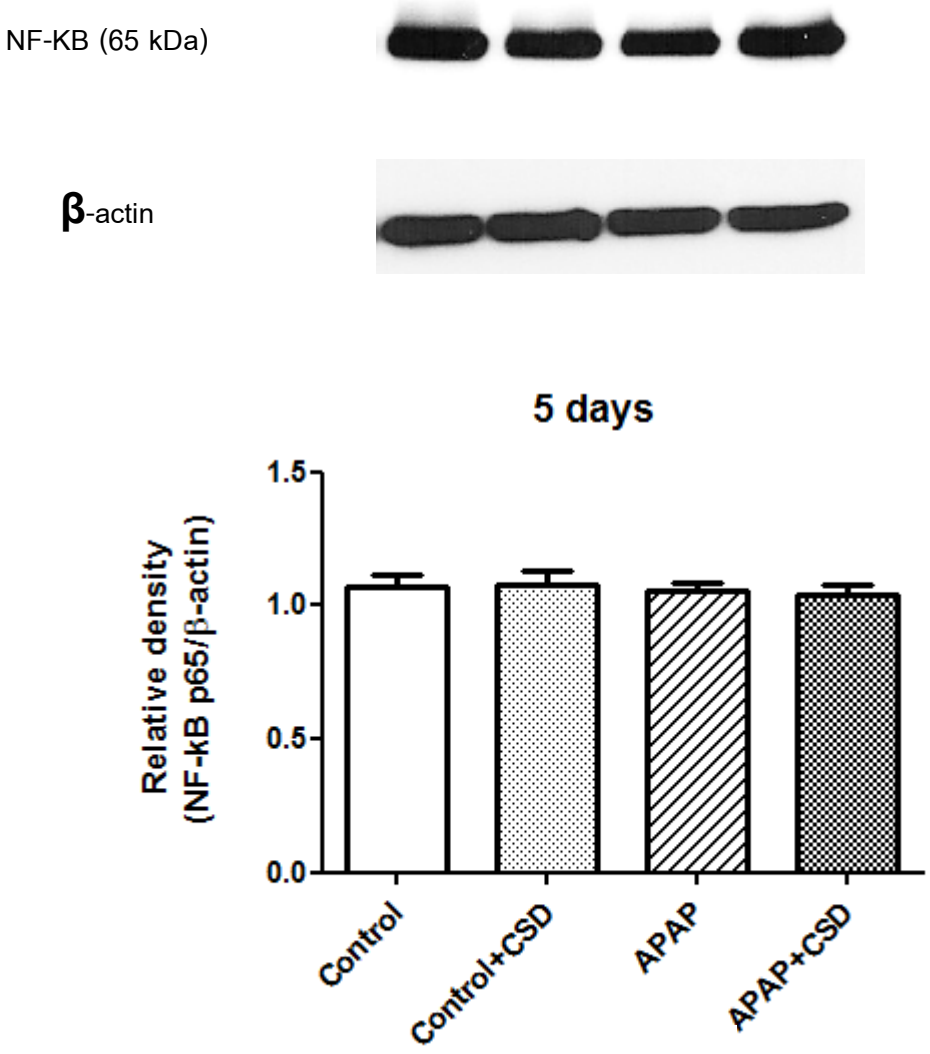


Figure 4-15 The effect of 5 days paracetamol treatment on CSD-induced an expression of the transcription factor NF-kB p65 in the cerebral cortex. Protein levels were analyzed by western blotting. Quantitative data are expressed as relative density to β-actin. The values represent the mean ± SD (n=4).

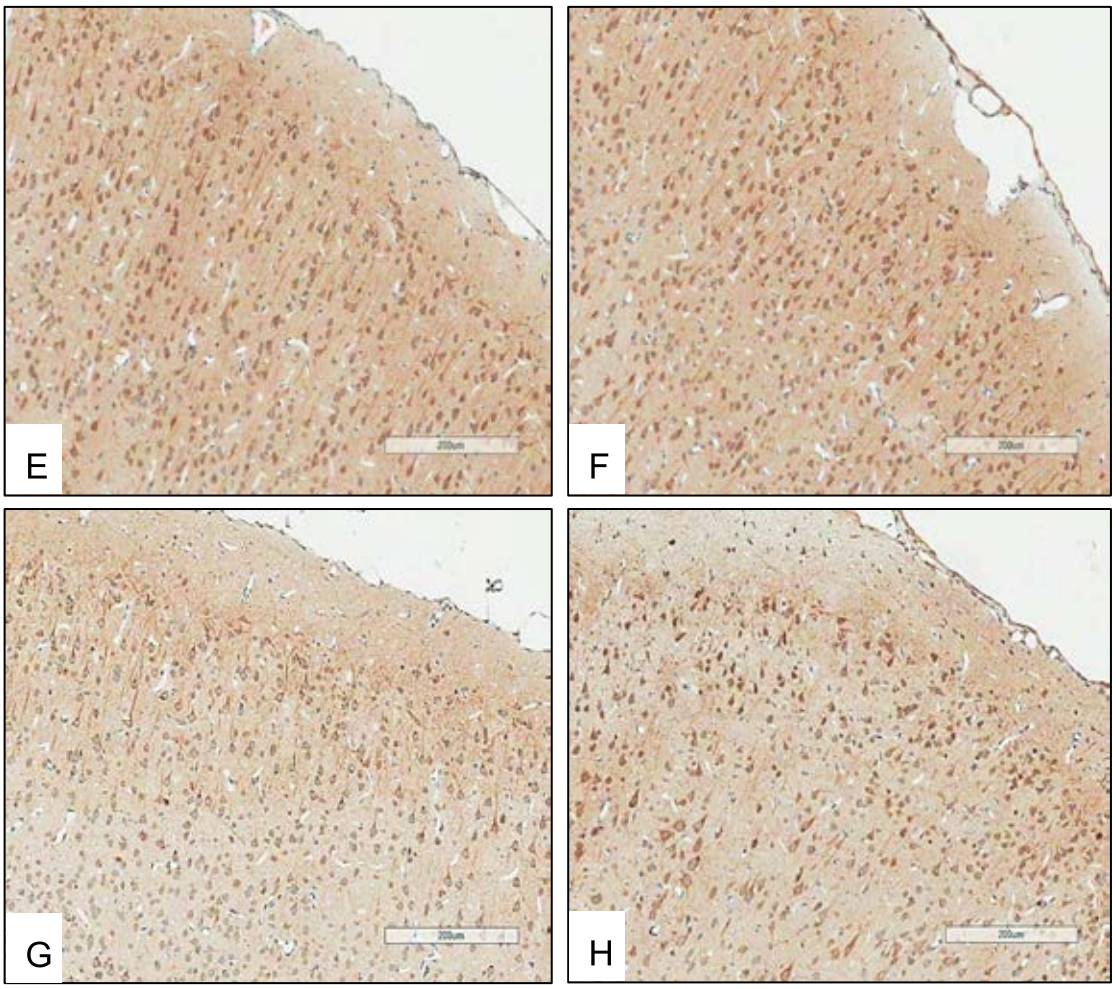


Figure 4-16 The photomicrograph shows localization of the NF-kB immunoreactive cells (dark brown cells) in the cerebral cortex at 5 days obtained from the control (E), control with CSD activation (F), paracetamol (G), and paracetamol with CSD activation group (H).

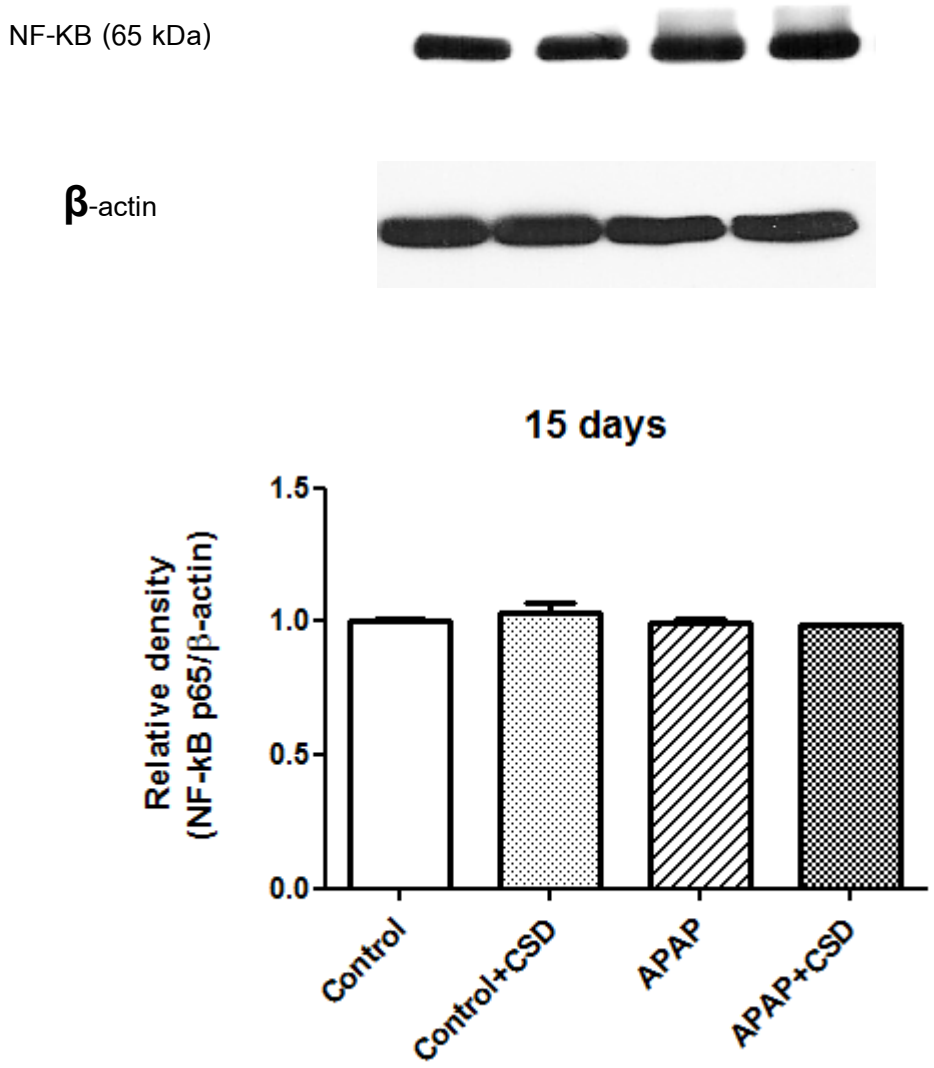


Figure 4-17 The effect of 15 days paracetamol treatment on CSD-induced an expression of the transcription factor NF-kB p65 in the cerebral cortex. Protein levels were analyzed by western blotting. Quantitative data are expressed as relative density to β-actin. The values represent the mean ± SD (n=4).

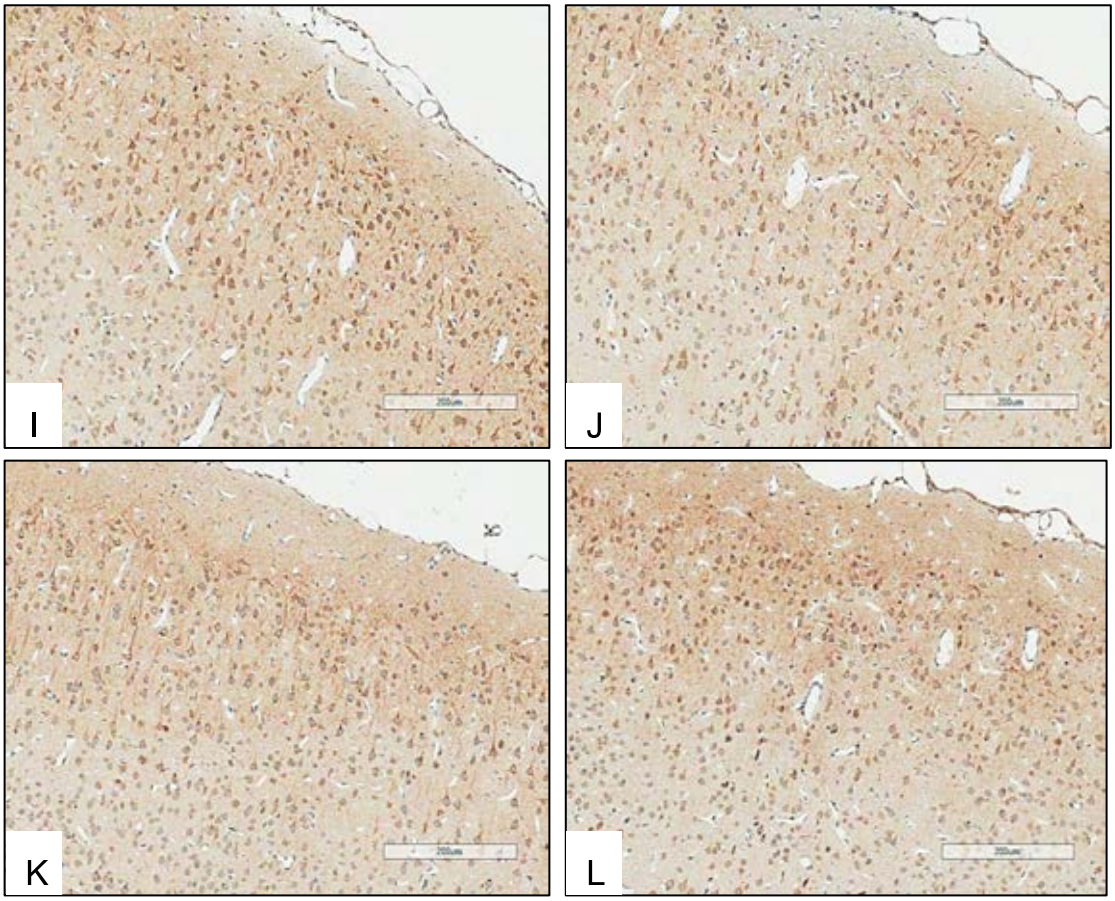


Figure 4-18 The photomicrograph shows localization of the NF-kB immunoreactive cells (dark brown cells) in the cerebral cortex at 15 days obtained from the control (I), control with CSD activation (J), paracetamol (K), and paracetamol with CSD activation group (L).

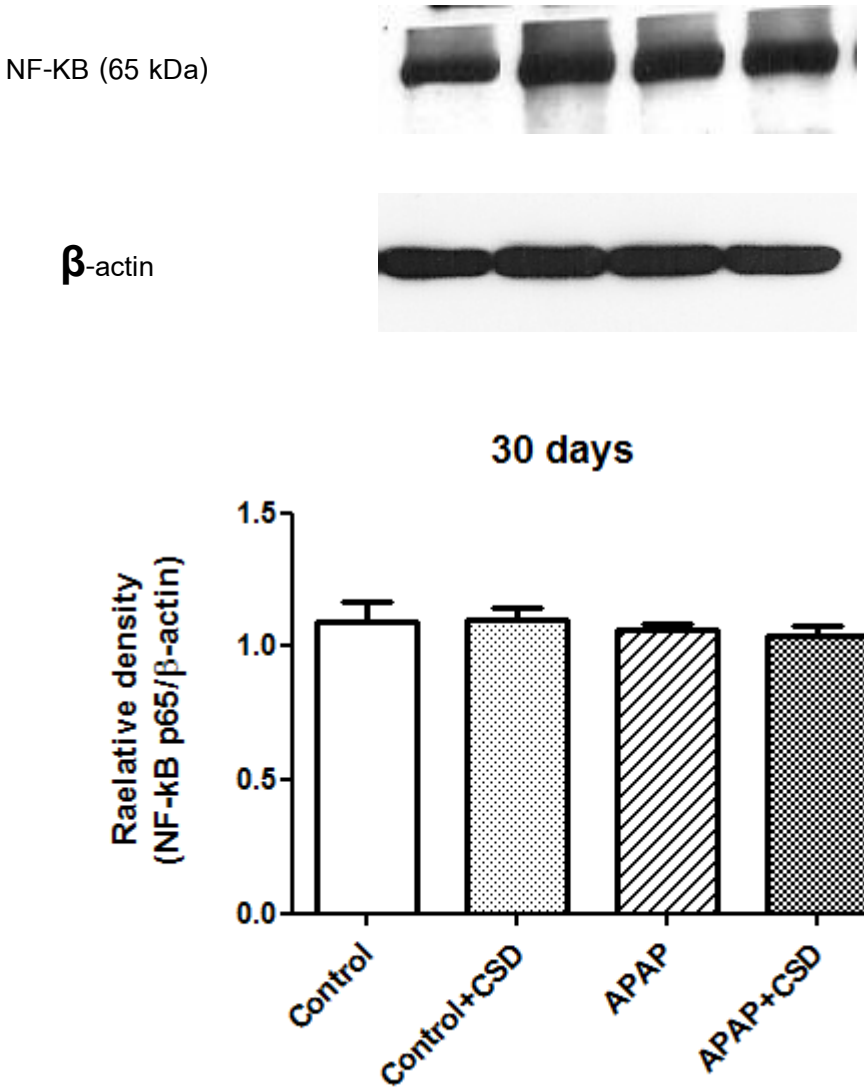


Figure 4-19 The effect of 30 days paracetamol treatment on CSD-induced an expression of the transcription factor NF-kB p65 in the cerebral cortex. Protein levels were analyzed by western blotting. Quantitative data are expressed as relative density to β-actin. The values represent the mean ± SD (n=4).

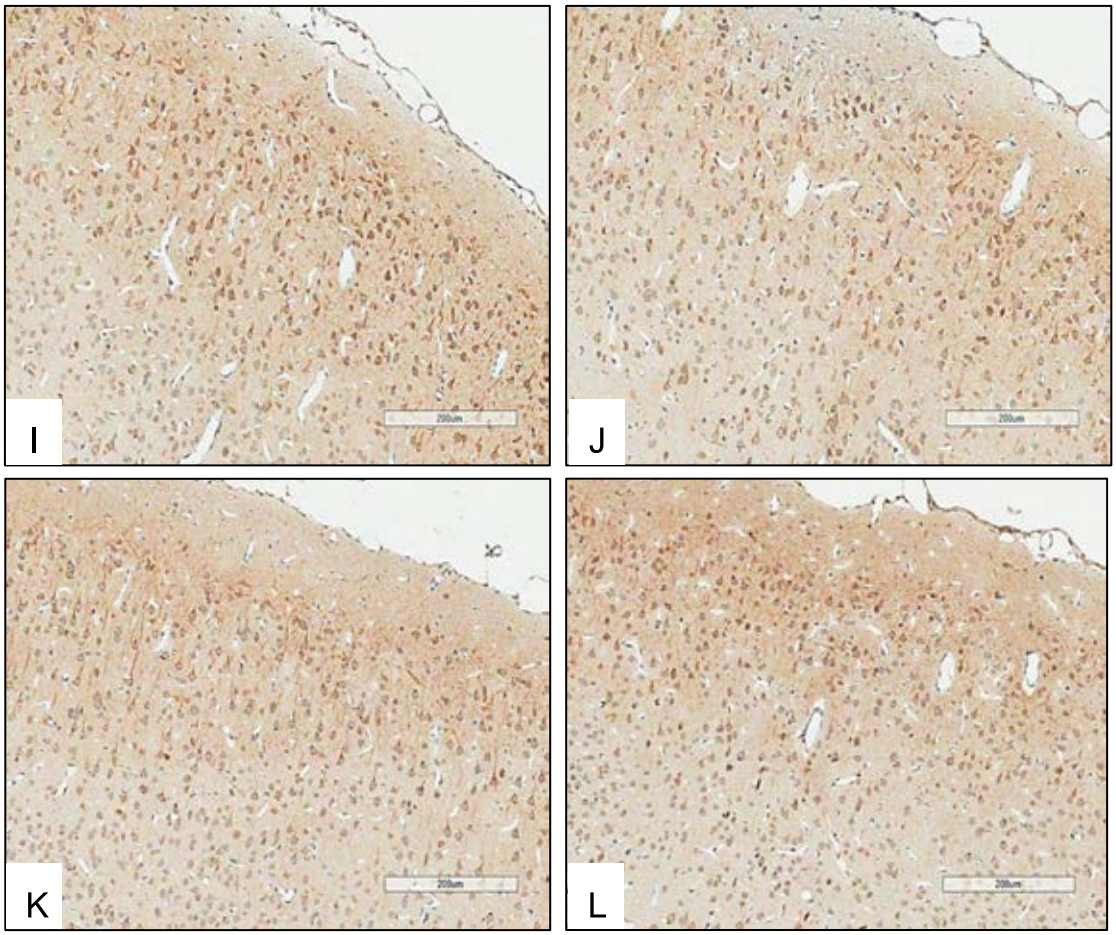


Figure 4-20 The photomicrograph shows localization of the NF-kB immunoreactive cells (dark brown cells) in the cerebral cortex at 30 days obtained from the control (M), control with CSD activation (N), paracetamol (O), and paracetamol with CSD activation group (P).

Table 4-4 Effect of paracetamol treatment at four different time points on the expression of transcription factor NF-kB in the rat's cerebral cortex.

Paracetamol administration (days)	Control (n=5)	Control + CSD (n=5)	Paracetamol (n=5)	Paracetamol + CSD (n=5)
0	81.50±13.45	82.15±11.09	87.04±13.03	86.91±11.97
5	83.32±12.98	80.13±12.96	81.97±13.04	83.03±12.13
15	79.09±10.97	76.91±9.78	74.91±10.05	73.92±14.11
30	77.15±15.01	76.43±9.96	73.34±9.07	71.54±13.01

The data are expressed as the mean ± SD.

4.4.2 Expression of phospho-NF-kB

The results of western blotting are shown in Figure 4-21, 4-22, 4-23 and 4-24. Induction of CSD by KCl application as well as the short term treatment of paracetamol (0 and 5 days) had no effect on the expression of the phosphorylated NF-kB. However, the expression of phospho-NF-kB p65 increased significantly compared with the control group when paracetamol was treated chronically (15 and 30 days). This expression also had significantly increased in the chronic paracetamol treated (15 and 30 days) in combination with CSD activation groups as compared with control group.

Further, when comparing the level of phosphorylated NF-kB expression between the paracetamol treated with and without CSD activation, only a significant difference in the level of expression was demonstrated for the duration of 15 days treatment. While there was no difference in the NF-kB activation with the duration of 30 days.

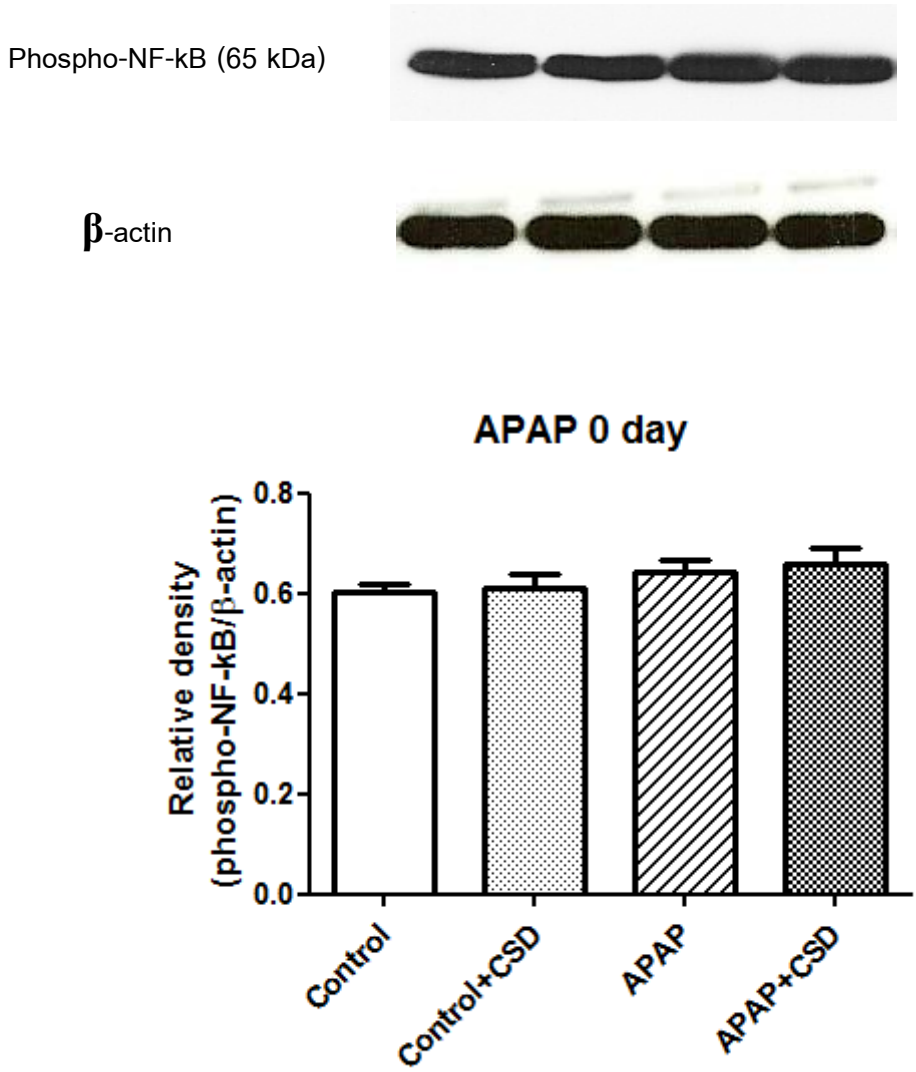


Figure 4-21 The effect of 0 days paracetamol treatment on CSD-induced an expression of the phospho-NF-kB p65 in the cerebral cortex. Protein levels were analyzed by western blotting. Quantitative data are expressed as relative density to β-actin. The values represent the mean ± SD (n=4).

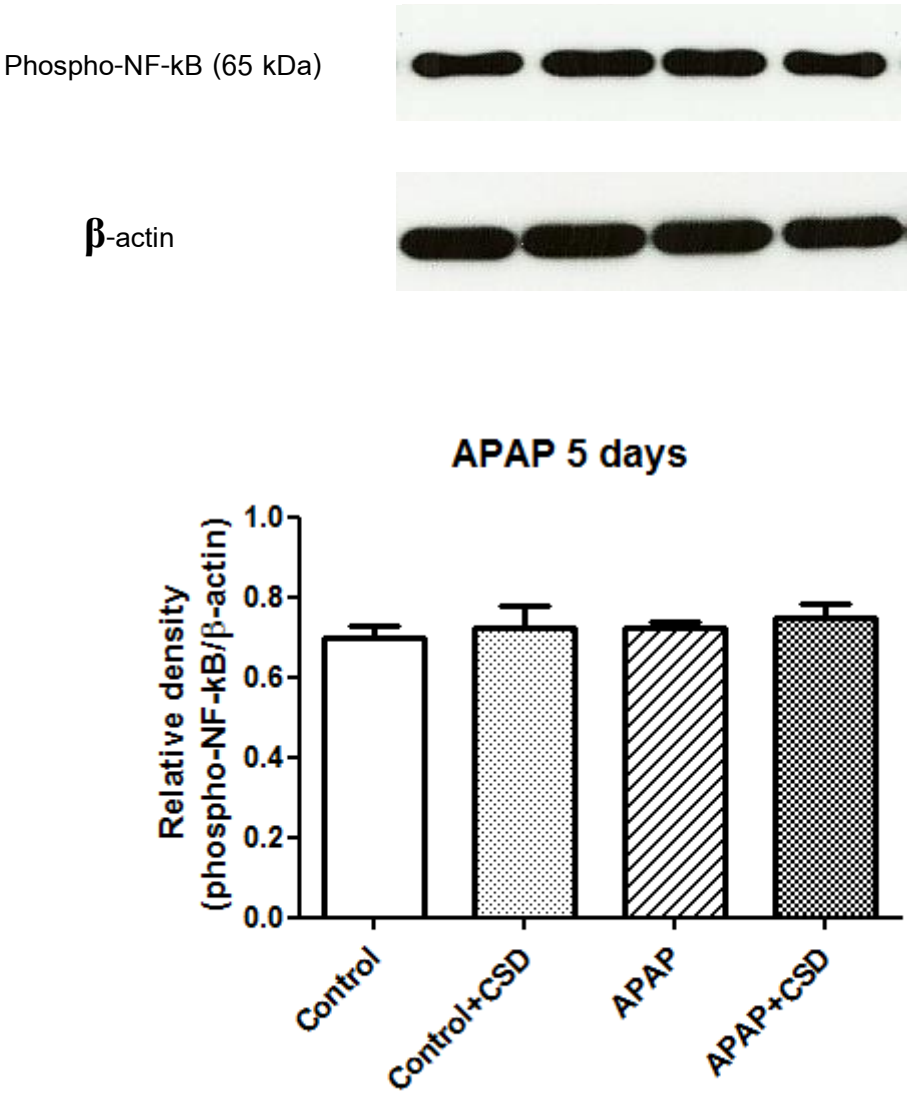


Figure 4-22 The effect of 5 days paracetamol treatment on CSD-induced an expression of the phospho-NF-kB p65 in the cerebral cortex. Protein levels were analyzed by western blotting. Quantitative data are expressed as relative density to β-actin. The values represent the mean ± SD (n=4).

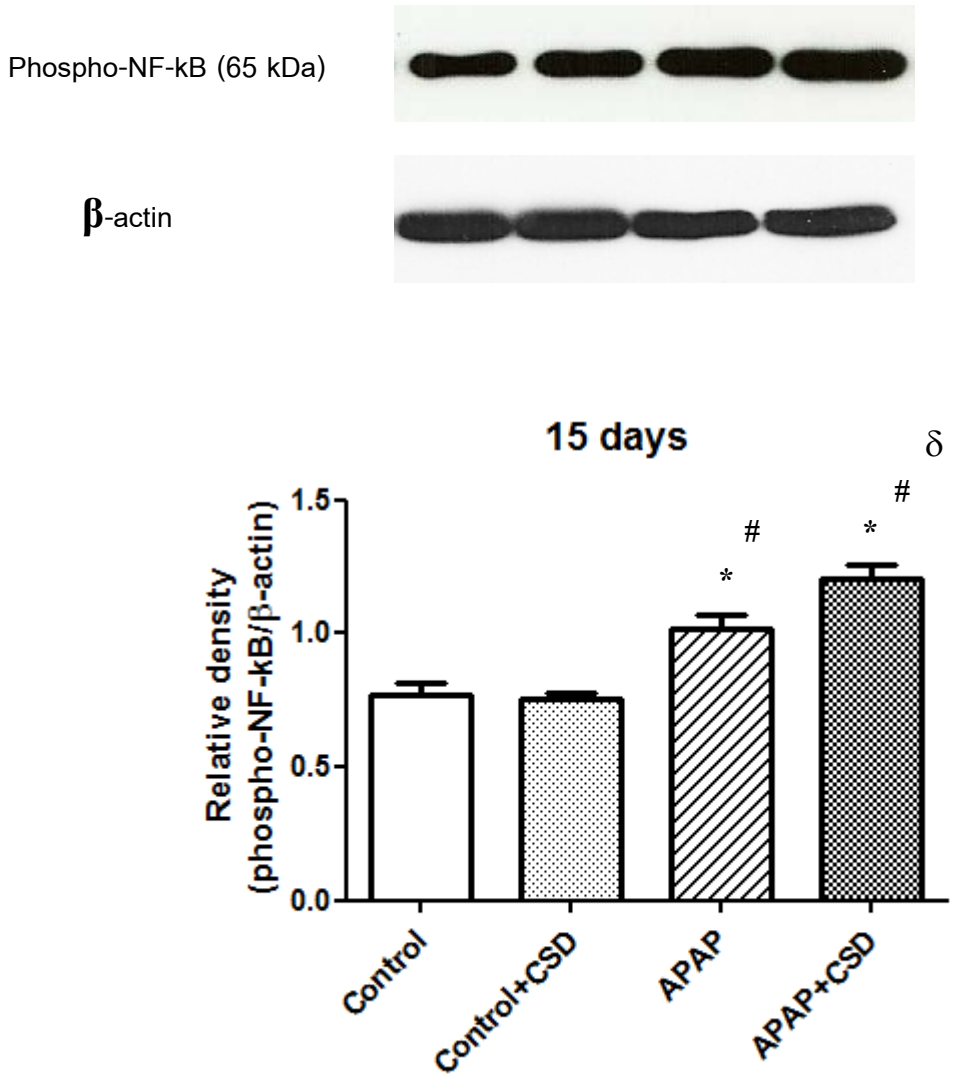


Figure 4-23 The effect of 15 days paracetamol treatment on CSD-induced an expression of the phospho-NF-kB p65 in the cerebral cortex. Protein levels were analyzed by western blotting. Quantitative data are expressed as relative density to β-actin. The values represent the mean ± SD (n=4). $^{*}p<0.05$ compared with the control group. $^{\#}p<0.05$ compared with the control with CSD activation group. $^{\delta}p<0.05$ compared with the paracetamol group.

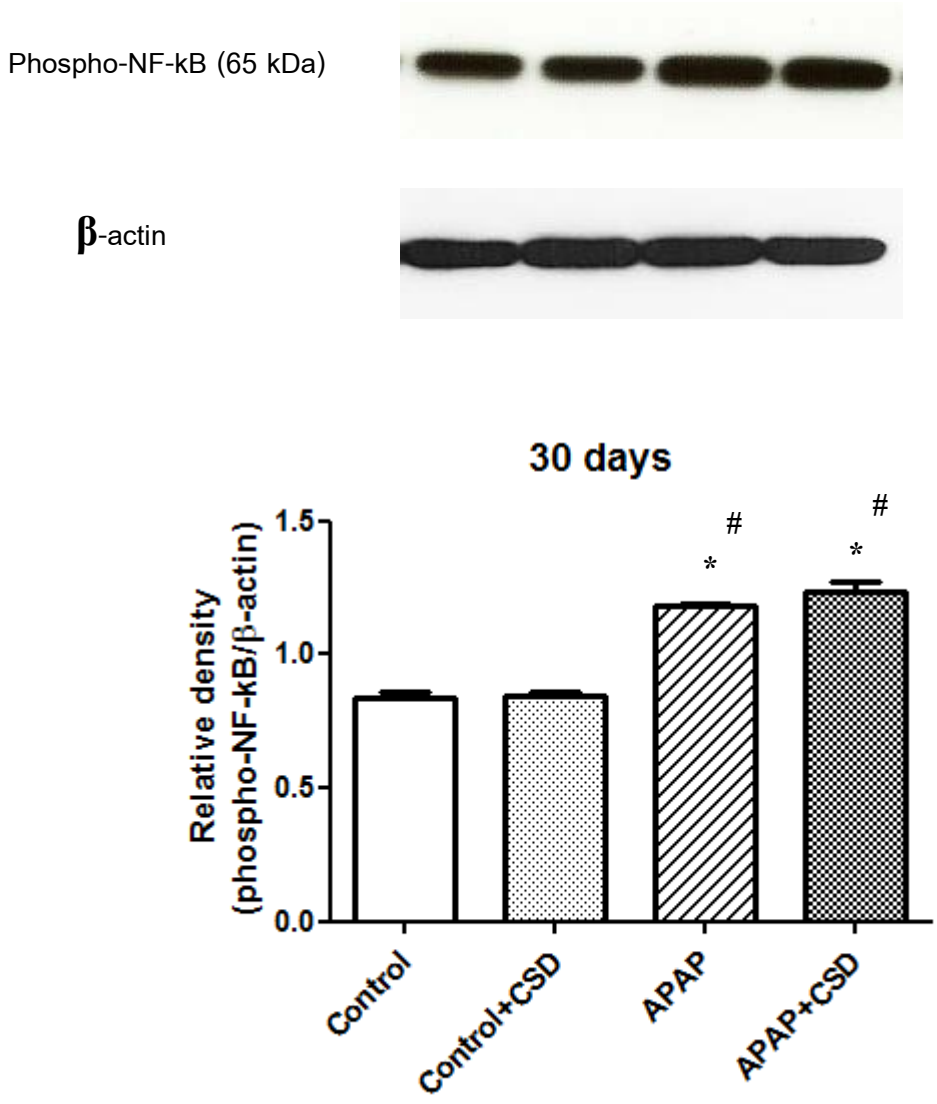


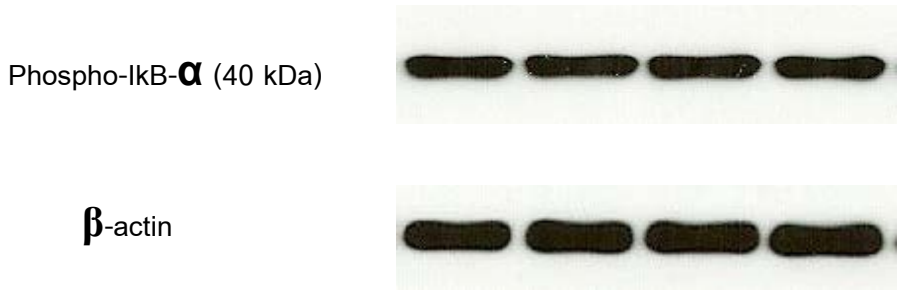
Figure 4-24 The effect of 30 days paracetamol treatment on CSD-induced an expression of the phospho-NF-kB p65 in the cerebral cortex. Protein levels were analyzed by western blotting. Quantitative data are expressed as relative density to β-actin. The values represent the mean ± SD (n=4). $^{*}p<0.05$ compared with the control group. $^{\#}p<0.05$ compared with the control with CSD activation group.

4.4.3 Expression of phospho-IkB

Western blot analysis of the phosphorylated-IkB p65 expression at the protein level in this study showed overall a similar pattern with the result from the study of an expression of phospho-NF-kB p65. The data are shown in figure 4-25, 4-26, 4-27 and 4-28. Based on the results, the short term treatment of paracetamol (0 and 5 days) with and without CSD activation had no effect on the expression of phospho-IkB. Though chronic paracetamol treatment (15 and 30 days) without CSD activation did significantly increase the expression as compared with those of the control group. The expression had also significantly increased through the chronic paracetamol treatment (15 and 30 days) in combination with CSD activation as compared with the control group.

Similar to the result of phosphorylated NF-kB expression, for only the chronic treatment with paracetamol of 15 days demonstrated a significant increase in the expression of phosphorylated-IkB p65 when comparing the paracetamol treated group and the paracetamol treated group with CSD activation.

The results from this part were associated with the result from the study of expression of phospho-NF-kB p65. These data indicate that the chronic treatment of paracetamol in both combination with and without CSD activation can activate the NF-kB signaling pathway in the brain.



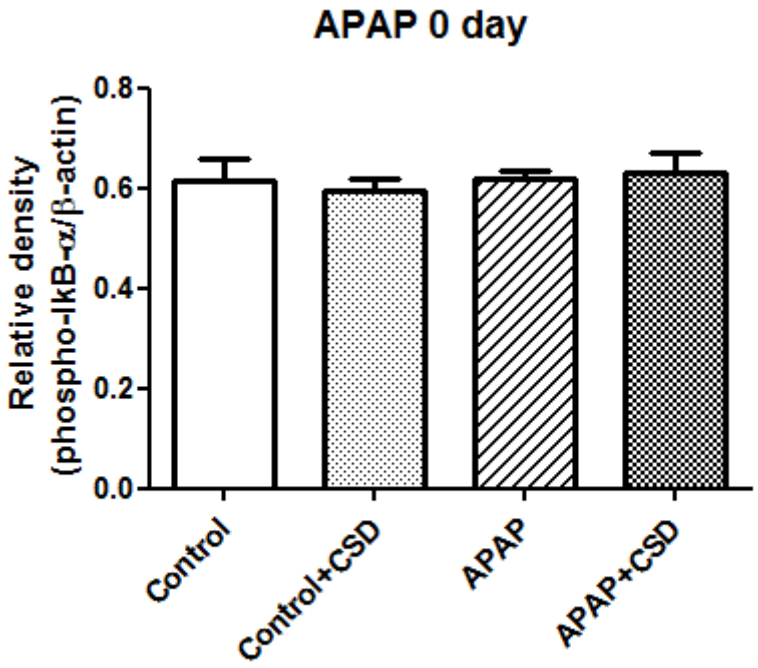


Figure 4-25 The effect of 0 days paracetamol treatment on CSD-induced an expression of phospho-IkB in the cerebral cortex. Protein levels were analyzed by western blotting. Quantitative data are expressed as relative density to β-actin. The values represent the mean ± SD (n=4).

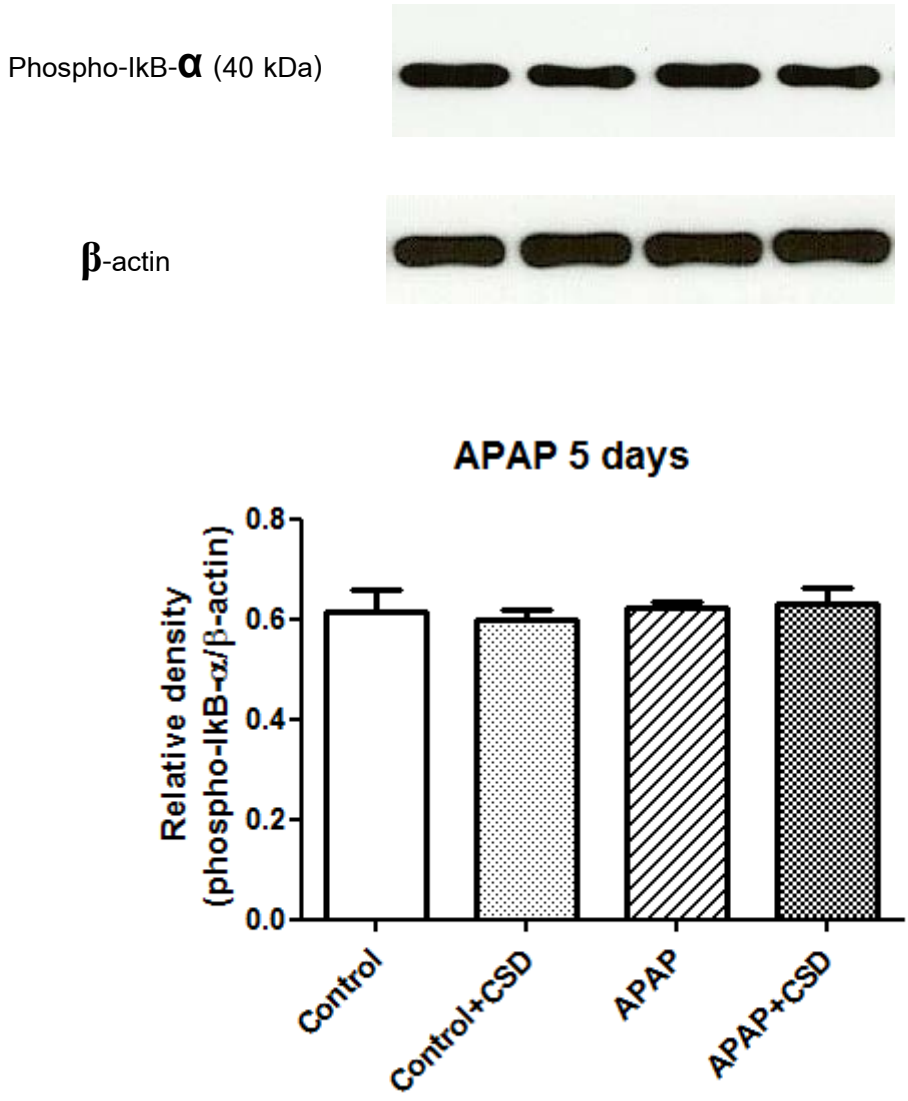


Figure 4-26 The effect of 5 days paracetamol treatment on CSD-induced an expression of phospho-IkB in the cerebral cortex. Protein levels were analyzed by western blotting. Quantitative data are expressed as relative density to β-actin. The values represent the mean ± SD (n=4).

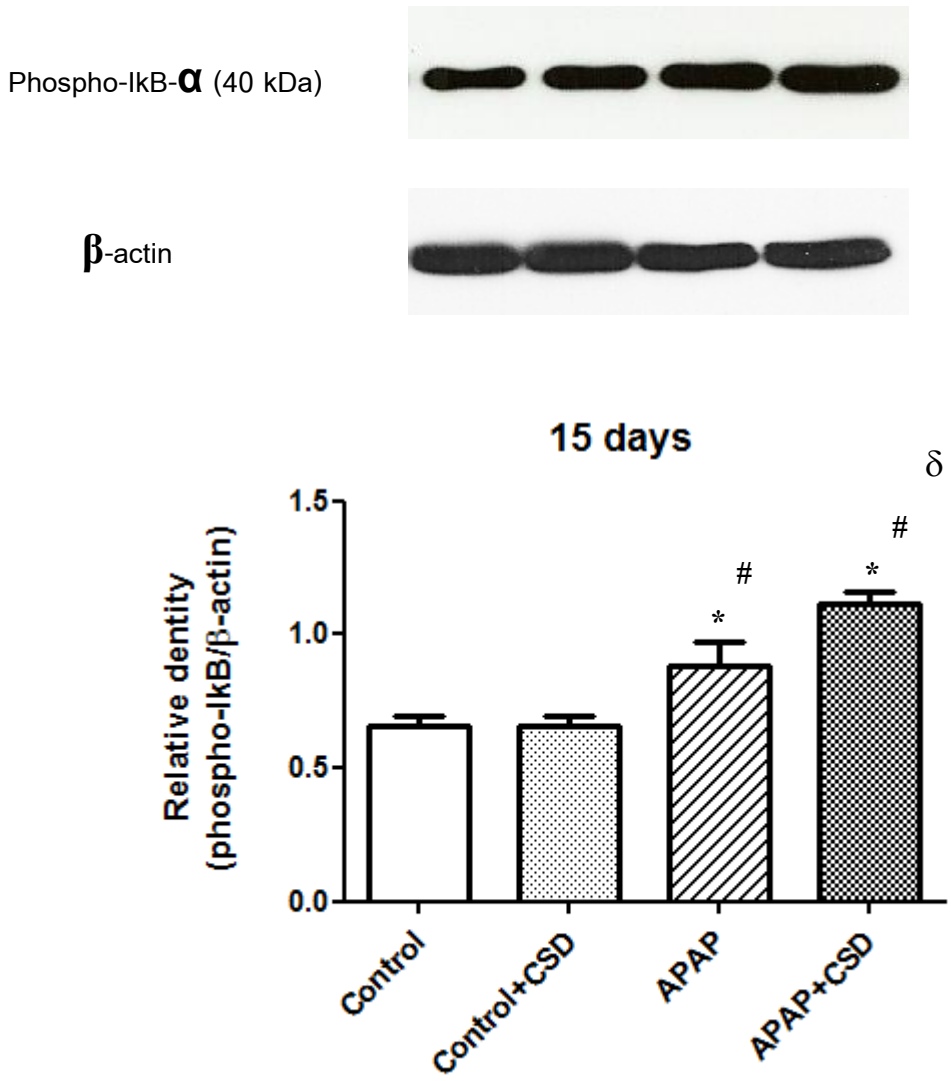
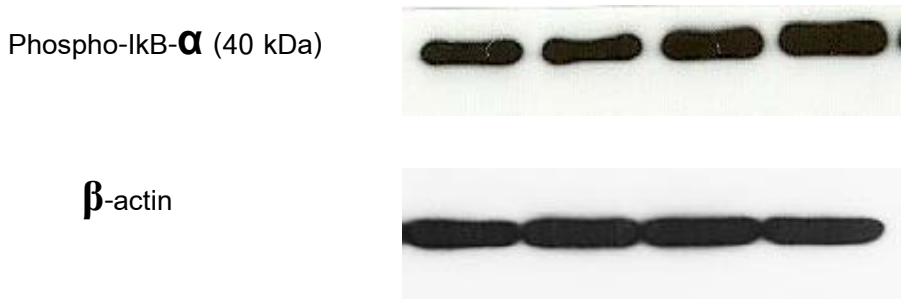


Figure 4-27 The effect of 15 days paracetamol treatment on CSD-induced an expression of phospho-IkB in the cerebral cortex. Protein levels were analyzed by western blotting. Quantitative data are expressed as relative density to β-actin. The values represent the mean ± SD (n=4). $^{*}p<0.05$ compared with the control group. $^{\#}p<0.05$ compared with control with CSD activation group. $^{\delta}p<0.05$ compared with paracetamol group.



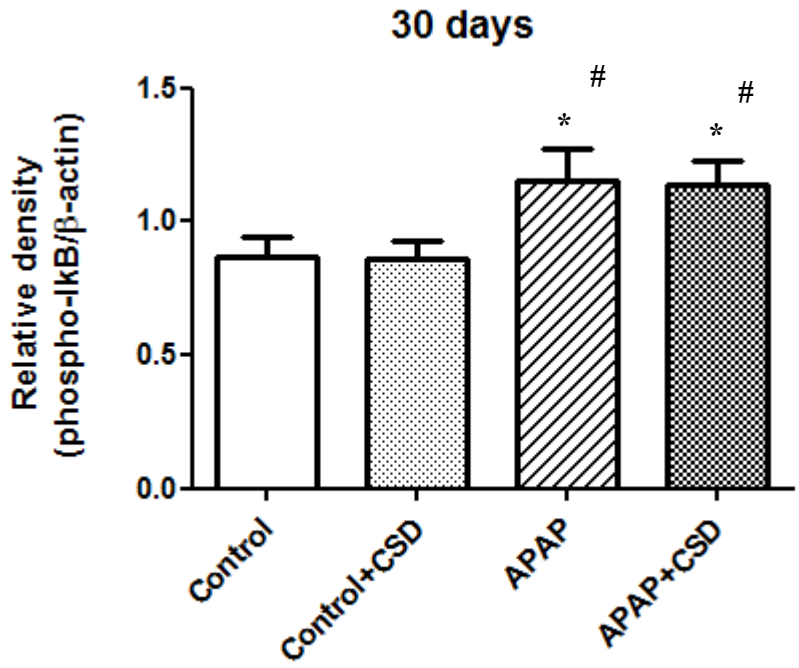


Figure 4-28 The effect of 15 days paracetamol treatment on CSD-induced an expression of phospho-IkB in the cerebral cortex. Protein levels were analyzed by western blotting. Quantitative data are expressed as relative density to β-actin. The values represent the mean ± SD (n=4). $^{*}p<0.05$ compared with the control group. $^{\#}p<0.05$ compared with control with CSD activation group.

4.5 Conclusion of the results

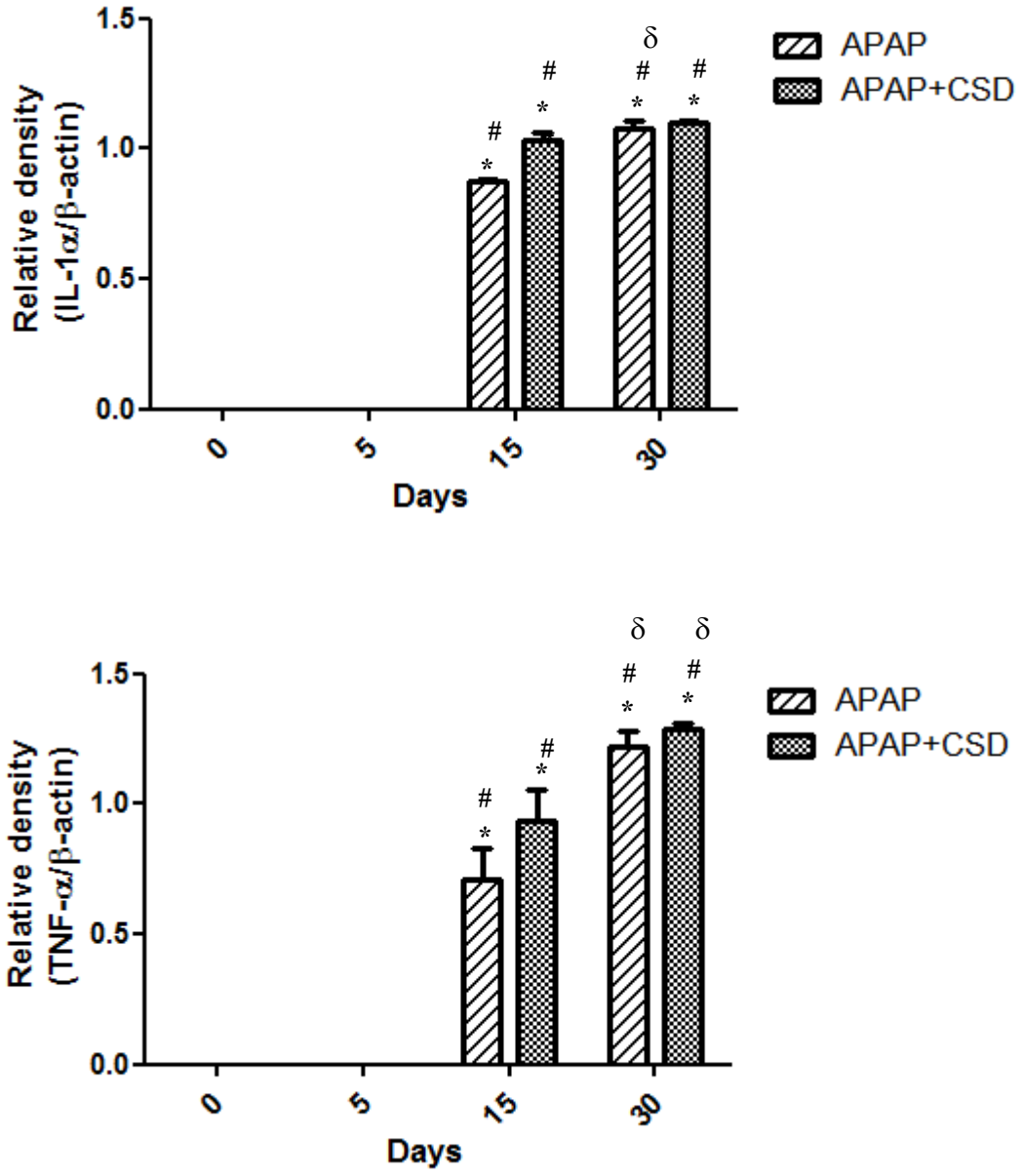


Figure 4-29 The expression of IL-1α and TNF-α in the cerebral cortex at four different time points. Protein levels were analyzed by western blotting. Quantitative data are expressed as relative density to β-actin. The values represent the mean ± SD (n=4). $^{*}p<0.05$ compared with 0 days treatment in the same group. $^{\#}p<0.05$ compared with 5 days treatment in the same group.

The result of this study has indicated that, in the condition with and without CSD activation, the expression of pro-inflammatory cytokines (both IL-1α and TNF-α) in the cerebral cortex were not affected by short term paracetamol treatment (0 and 5 days). However, these cytokines were increased through the course of chronic paracetamol treatment (15 and 30 days).The effect of chronic paracetamol treatment on the pro-inflammatory cytokines production is at least mediated via the activation of the NF-kB signaling pathway.

CHAPTER V

DISCUSSION

The results obtained from this study show that the brain responds differently to acute and chronic paracetamol exposure. Short term paracetamol exposure, acute paracetamol treatment at a period of 0 and 5 days, has no effect on the expression of pro-inflammatory cytokines IL-1α and TNF-α. The CSD activation without paracetamol treatment does not affect the expression of both cytokines. However, long term exposure, the paracetamol treatment for 15 and 30 days, can induce an increase in the IL-1α and TNF-α expression in the cerebral cortex. Our result also indicates that the enhancement of the production of those cytokines might mediate via the activation of the NF-kB signaling pathway.

Paracetamol is the most widely used over-the-counter medication as an antipyretic and analgesic drug [1]. It became one of the most popular drugs used for treatment of pain and several types of headaches because of various properties including low price, high availability without prescription, and low impact of side effects. Because of this, the risk exists that patients who suffer from chronic pain or migraine will use paracetamol as a chronic treatment.

Paracetamol, despite being classified as a non-steroidal anti-inflammatory drugs (NSAIDs), demonstrates the dual effect in inflammatory processes. Several studies in mice have reported that a single dose of paracetamol (300 mg/kg i.p.) could induce an increase in the expression of pro-inflammatory mediators, interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), as well as the anti-inflammatory cytokine, IL-10 [12]. Similar to the results in the transgenic mice model, paracetamol (300 mg/kg i.p.) increased the expression of IL-1β and IL-10 [44]. Because it can induce a series of both pro-inflammatory and anti-inflammatory cytokines, its role in the inflammation process is still controversial.

Most of the studies related to paracetamol have indicated the protective effect of this drug on various pathological conditions [1, 5-6]. With therapeutic doses, the side effect of this drug is hardly reported. Hepatotoxicity was close to the only adverse effect reported after administration of an acute overdoses or chronic treatment with higher doses than that in the

therapeutic range [7]. However in the last decade, few studies have reported the non-beneficial effect of this drug on several organs including lung [27], neuron [8], and the brain [11]. Studies in rats with CSD, an animal model for migraine headache, demonstrated that chronic paracetamol treatment (dose 200 mg/kg i.p. for 30 days) could increase the cortical neuron activities compared those observed in the control group [10-11]. Their results indicated that the chronic paracetamol treatment could alter the trigeminovascular nociceptive system. They suggested that chronic paracetamol treatment might sensitize the trigeminovascular nociception in both the peripheral (which involves both vascular and neural compartments) and central pathways. However, the mechanism underlying the deterioration of the trigeminovascular nociceptive system has not been clarified yet.

In this study, the dose of paracetamol which we had selected is the same dose with the previous study (200 mg/kg BW). We have found the same result which is that the selected dose did not cause any toxic effect to the hepatocyte, The levels of three liver enzymes, indicating liver toxicity from the rats with the longest treatment time (30 days) were still in the normal range. With this part of the results, it means that all alterations observed in paracetamol treatment are not associated with hepatotoxicity. Our results have demonstrated that short term treatment with paracetamol did not have any effect on the inflammatory cytokine production in the brain. This result indicated that paracetamol treatment is safe in short term exposure. However, we have discovered that whether in combination with CSD activation or not, the chronic treatment with paracetamol (more than 15 days treatment) could induce the pro-inflammatory cytokine expression (IL-1α and TNF-α) in the cerebral cortex.

This result is correlated well with the previous study in the headache animal model, in which short term or pretreatment with paracetamol (at the same dose with what we used in this study) demonstrated the beneficial effect of this drug. It could attenuated the CSD induced excitation of cortical neurons as well as the activation of second order neurons in the trigeminal nucleus caudalis (TNC). While with long term treatment (30 days) of paracetamol, the ability of this drug in inhibition of cortical neurons and TNC excitation induced by CSD were significantly reduced [10-11]. In this study, we have found that, long term exposure to paracetamol (15 and 30 days treatment) led to an increase in the expression of pro-inflammatory cytokines in the brain. Interestingly, the enhancement of those pro-inflammatory cytokine expressions by CSD

activation was observed only in the group of 15 days treatment while in the 30 days treatment group the activation of CSD could not add up the expression of pro-inflammatory cytokines in the brain. However, when comparing the levels of these pro-inflammatory cytokines between the group with paracetamol treatment for 15 days and 30 days, the level of these cytokines was significantly higher in the group with 30 days paracetamol treatment. Based on these results it could be suggest that 30 day treatment with paracetamol at the dosage used in this study could maximally induce the pro-inflammatory cytokines production in the rat brain, so the CSD activation fail to add up the expression of these cytokines as observed in this study.

It had previously been described that chronic paracetamol treatment could lead to an increase in neuronal excitability and cortical excitability in the cerebral cortex. The sensitization of trigeminovascular nociception is one of the mechanisms that explains the hyperexcitability of these cortical neurons [10-11]. The inflammatory cytokines have long been known as a mediators involved in the migraine pathophysiology. The increment of inflammatory cytokine level has been reported in studies from both the clinical and animal headache model (34, 37, 45]. Taken the role of neurogenic inflammation in migraine pathophysiology together with the results obtained from this study it can be suggested that the increment in the level of the inflammatory cytokines in the brain causing by the chronic paracetamol treatment is, at least, one mechanism underlying the cortical neuron hyperexcitability observed in previous study.

Based on the results obtained from this study, the adverse effect of this drug treatment on the brain seems to be depending on the duration of the treatment. The short term treatment had no effect on the pro-inflammatory cytokine production while long term treatment could increase the pro-inflammatory cytokine production in the brain. Actually, in the last decade, several adverse effects of this drug when using as a long term treatment were gradually reported on several organs. In 2007, 4 year prospective cohort study of Forman and coworkers had reported an increased relative risk for incident hypertension in men who took paracetamol for 6-7 days/week compared with nonusers [46]. Further the study by Sudano et al. in 2011 had revealed results in the same direction. They found that adding a daily treatment of paracetamol at the therapeutic dose on the regular treatment (1 g TID) in patients with coronary artery disease for 2 weeks could significantly increase blood pressure in both systolic and diastolic pressure [47]. Last year, Posadas et al. has reported for the first time that treatment with paracetamol at the

doses which lower than those required to produce hepatotoxicity could induce the neurotoxic effect both *in vivo* and *in vitro* studies [25]. However, the adverse effect caused by chronic treatment with this drug on the production of pro-inflammatory cytokines in the brain has just been discovered by our group.

Paracetamol is mainly metabolized in the liver via conjugation with glucuronic acid, sulphuric acid, or cysteine and then excreted into the urine. Only a small fraction is metabolized by cytochrome P-450 resulting in the formation of NAPQI [23-24]. The NAPQI, will be quickly captured by glutathione (GSH) and excreted into the urine as well. The CYP2E1, the most important isoform of cytochrome P-450, is the most abundant in the liver. Accumulative evidence have demonstrated that the expression of CYP2E1 is not only expressed in the liver but also expressed in other organ including the brain (neurons, astrocytes, and endothelial cells) [8, 48-49]. Although the expression of CYP2E1 in the brain is present at low levels, its activity is actually higher than those observed in the liver [50]. Studies in both rodent and human had demonstrated that paracetamol can freely cross the blood-brain barrier (BBB) [51]. Base on this, it means that after reaching the cerebral circulation, paracetamol can pass through the BBB and be metabolized into NAPQI by those cells which express CYP2E1. NAPQI is a toxic substance for the brain, thus it will be detoxify quickly by brain GSH. In case of a high concentration of paracetamol, the excessive level of NAPQI will directly bind to cellular proteins leading to the cellular damage and death [8, 24, 52]. In our study, the steady moderate level of this drug in the brain causing by the chronic treatment will lead to the continuous production of NAPQI which subsequently lead to the depletion of GSH. The depletion of GSH in the brain can induce the oxidative stress which will finally cause cell damage as well.

The nuclear factor kappa B (NF-kB) is a transcription factor which plays a key role in the response to both inflammation and oxidative stress. NF-kB is found in almost all animal cell types and is involved in cellular responses to stimuli such as pro-inflammatory cytokine or oxidative stress. In this study, the activation of the NF-kB signaling pathway (an increase in the expression of phorpho-NF-kB as well as the IkB) was clearly demonstrated in the animal with chronic paracetamol treatment (15 and 30 days treatments). In resting state, NF-kB is located in cytoplasm interacting with the inhibitory protein (IkB). Activation of the NF-kB signaling pathway will result in phosphorylation of IkB and subsequently the translocation of NF-kB from

cytoplasm to the nucleus to activate transcription of the target gene such as IL-1α and TNF-α [42]. The activation or inactivation of NF-kB is regarded as a key factor regulating the cellular pro-inflammatory and anti-inflammatory balance [43].

In this present study, taken the observation of the increment of pro-inflammatory cytokine together with the activation of the NF-kB signaling pathway demonstrated in animals with chronic paracetamol treatment, we can strongly suggest that the increase of the pro-inflammatory cytokines via the activation of the NF-kB signaling pathway can be one explanation for the abnormal responses of the cortical neurons observed in CSD animal model.

This study demonstrates for the first time that even though the short term treatment with paracetamol has no effect on the pro-inflammatory cytokine production in the brain, the long term treatment with the same drug can induce a significant increase in the expression of pro-inflammatory cytokines in the cerebral cortex. Noteworthy, even only 15 days treatment with this drug can induce this adverse effect to the brain, therefore the general believe in using paracetamol as a drug of choice might need to be reconsidered.

CHAPTER VI

CONCLUSION

The present study demonstrates the different effect of acute and chronic paracetamol treatment on the expression of pro-inflammatory cytokines in the brain. While short term paracetamol treatment is safe, chronic paracetamol treatment leads to an increase in pro-inflammatory cytokines (both IL-1α and TNF-α) production. Based on these results, the increment of the pro-inflammatory cytokines might mediate via the activation of the NF-kB signaling pathway.

It should be noted that, paracetamol, drug of choice for people who have pain and headaces, is not always acting as a safe drug anymore. People who take paracetamol for more than 1 week should be aware of the effect of paracetamol especially using this drug in the combination with migraine attack.

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APPENDIX

APPENDIX

REAGENT AND PREPARATIONS

Stock Solutions

10x SDS-PAGE running buffer volume 1 liter : [250 mM Tris-HCl, 1.92 mM Glycine, 1% SDS]

- Tris-HCl (MW 121.14) 30.28 g - - > final conc. 25 mM
- Glycine (MW 75.07) 144.13 g - - > final conc. 192 mM
- Sodium dodecylsulfate (SDS) 10 g - - > final conc. 0.1%

Add dH_2O up to 1000 ml

10x Transfer buffer volume 800 ml : [250 mM Tris-HCl, 1.92 mM Glycine]

- Tris-base (MW 121.14) 30.28 g
- Glycine (MW 75.07) 144.13 g

Add dH_2O 800 ml

10x Tris-buffer Saline (TBS) volume 1 liter : [100 mM Tris-HCl (pH 7.5), 150 mM NaCl]

- Tris-HCl (MW 121.14) 121.1 g
- NaCl (MW 58.4) 90 g

Add dH_2O almost ~ 900 ml - -> adjust pH to 7.5 by HCl

Add dH_2O up to 1000 ml

10x TBS buffer volume 1 liter : [50 mM Tris-HCl (pH 7.6), 150 mM NaCl]

-Tris-HCl (MW 121.14) 60.57 g
- NaCl (MW 58.4) 87.6 g

Add dH_2O almost ~800 ml - -> adjust pH to 7.6 by HCl

Add dH_2O up to 1000 ml

10% SDS (w/v) volume 100 ml :

- sodium dodecylsulfate (SDS) 10 g

Add dH_2O up to 100 ml

Store at room temperature

4x Running Gel buffer volume 200 ml : **[1.5 M Tris-HCl, pH 8.8]**

- Tris-HCl (MW 121.14) 36.3 g

Add dH_2O almost ~ 150 ml - -> adjust pH to 8.8 by HCl

Add dH_2O up to 200 ml

Store at 4ºc

4x Stacking Gel buffer volume 50 ml : **[0.5 M Tris-HCl, pH 6.8]**

- Tris-HCl (MW 121.14) 3 g

Add dH_2O almost ~ 40 ml - -> adjust pH to 6.8 by HCl

Add dH_2O up to 50 ml

Store at 4ºc

Coomassie blue staining volume 1 liter : [0.1%(w/v) Coomassie Brilliant Blue R250, 40% methanol, 10% glacial acetic acid]

- Coomassie Brilliant Blue R250 1 g
- Methanol 400 ml

Stirr ~3 hr until dissolved. Then add:

- Glacial acetic acid 100 ml

Add dH_2O up to 1000 ml * filter before store*

Store at room temperature

Destaining solution I volume 1 liter : [40 % methanol, 10 % acetic acid]

- Methanol	400	ml
-Acetic acid	100	ml

Add dH_2O up to 1000 ml

Store at room temperature

Destaining solution II volume 1 liter : [10 % Methanol, 5 % Acetic acid]

- Methanol	100	ml
-Acetic acid	50	ml

Add dH_2O up to 1000 ml

Store at room temperature

Working Solutions

Ice-cold Tris buffer (5mM Tris-HCl, pH 7.4 autoclave) volume 500 ml :

-Tris- base (MW 121.14) 0.3 g

Add dH_2O almost ~ 400 ml - -> adjust pH to 7.4 by HCl

Add dH_2O up to 500 ml

Store at 4°c

4X SDS Protein Sample Buffer (4X Loading dye) volume 10 ml : [240 mM Tris-HCl (pH 6.8), 40% Glycerol, 8% SDS, 0.04% Bromophenol blue, 5% β-mercaptoethanol]

-1 M Tris-HCl (pH 6.8)	2.4	ml
-100 % Glycerol	4	ml
-SDS	0.8	g
-1% Bromophenol blue	0.4	ml
- β-mercaptoethanol	0.5	ml
Add dH_2O	3.1	ml

1x SDS-PAGE running buffer volume 1 liter : [final conc. 25 mM Tris-HCl, 192 mM Glycine, 0.1% SDS]

- 10x SDS-PAGE running buffer 100 ml

Add dH_2O up to 1000 ml

Store at 4ºc

1x Transfer buffer volume 1 liter :

- 10x Transfer buffer 80 ml
- 100% methanol 200 ml
-Add dH_2O 720 ml

Store at 4ºc

Tris-buffer Saline (TBS) volume 1 liter : [100 mM Tris-HCl (pH 7.5), 150 mM NaCl]

- Tris-HCl (MW 121.14) 12.11 g
- NaCl (MW 58.4) 9 g

Add dH_2O almost ~ 900 ml - -> adjust pH to 7.5 by HCl

Add dH_2O up to 1000 ml

Store at room temperature

TBS with 0.1%Tween-20 (TBST) volume 1 liter :

- TBS 1000 ml
- Tween-20 1 ml

Store at room temperature

BIOGRAPHY

Name	Miss Chattraporn Chantong
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