Antidepressant-like Effects of Total Glycosides of Peony and its Possible Mechanisms

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A Thesis Submitted in Partial Fulfilment

of the Requirements for the Degree of Doctor of Philosophy

in

Chinese Medicine

The Chinese University of Hong Kong

August 2010

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Abstract of thesis entitled:

Antidepressant-like Effects of Total Glycosides of Peony and its Possible Mechanisms Submitted by Mao Qingqiu

for the degree of Doctor of Philosophy in Chinese Medicine

at The Chinese University of Hong Kong in August, 2010

Abstract

The root of *Paeonia lactiflora* Pall. (Family: Ranunculaceae), commonly known as peony, is a component herb of many traditional formulae for the treatment of depression-like disorders. Previous studies have demonstrated the antidepressive effect of peony extract in mouse models of depression. Total glycosides of peony (TGP) is regarded as the major active ingredients of peony. The present study aims to confirm the antidepressive potential of TGP and evaluate its action mechanisms.

The antidepressant-like effect of TGP was firstly evaluated by the behavioral despair test, forced swim test and tail suspension test. The results showed that intragastric administration of TGP caused a significant reduction of immobility time in both forced swim and tail suspension tests in mice. TGP treatment also significantly reduced the duration of immobility time in the forced swim test in rats.

Secondly, the antidepressant-like effect of TGP was evaluated by a rat model of depression

induced by chronic unpredictable mild stress (CUMS). The results showed that a 5-week CUMS caused depression-like behavior in rats, as indicated by a significant decreases in sucrose consumption (assessed by sucrose preference test) and locomotor activity (assessed by open-field test), and an increase in immobility time (assessed by forced swim test). Intragastric administration of TGP during the five weeks of CUMS procedure significantly suppressed these behavioral changes induced by CUMS.

Thirdly, the neuroprotective effects of TGP on CUMS-treated rats and its possible mechanisms were investigated. The results showed that treatment with TGP for 5 weeks produced neuroprotective effects on the hippocampus of CUMS-treated rats. This effect was associated with the attenuation of hypothalamic-pituitary-adrenal axis hyperactivation (characterized by a decreased serum corticosterone level and an increased hippocampal glucocorticoid receptor expression), an inhibition of oxidative stress, and up-regulation of neurotrophins such as brain-derived neurotrophic factor and neurotrophin-3 in the hippocampus.

Finally, the neuroprotective effects of TGP against corticosterone-induced neurotoxicity in rat pheochromocytoma (PC12) cells, an *in vitro* experimental model of depression were studied. The results showed TGP treatment dose-dependently protected the cells against corticosterone-induced toxicity. The cytoprotection afforded by TGP treatment was shown to be associated with an enhanced antioxidant activity, and increased expressions of neurotrophins including brain-derived neurotrophic factor, nerve growth factor and neurotrophin-3.

Taken together, the results confirmed the antidepressant-like effect of TGP. The antidepressive action of TGP may be mediated by the modulation of the hypothalamic-pituitary-adrenal axis function, the inhibition of oxidative stress, and the up-regulation of neurotrophins, thereby leading to the neuroprotective effects.

論文摘要

白芍是中醫臨床治療抑鬱症的常見中藥之一。課題組之前的藥理實驗也表明白芍提取物有抗抑鬱症作用。白芍總苷是白芍的有效成分，本論文將研究白芍總苷的抗實驗性抑鬱症作用，並探討其可能作用機制。

首先應用強迫游泳實驗和懸尾應激實驗檢測白芍總苷的抗抑鬱作用。實驗結果表明白芍總苷口服給藥能顯著縮短強迫游泳及懸尾應激小鼠的不動時間。白芍總苷口服給藥同時還能顯著縮短強迫游泳大鼠的不動時間。

其次，應用未預知慢型應激抑鬱症大鼠模型進一步驗證白芍總苷的抗抑鬱作用。實驗結果表明 5 周未預知慢型應激能導致大鼠出現抑鬱症狀，表現爲與正常大鼠比較：未預知慢型應激組大鼠的糖水消耗量顯著減少；未預知慢型應激組大鼠的自主活動明顯減少；未預知慢型應激組大鼠的強迫游泳時間明顯增加。白芍總苷與應激同時口服給藥 5 周能顯著抑制由未預知慢型應激引起的大鼠行爲學變化。

在確認白芍總苷具有抗抑鬱症作用的研究基礎之上，應用未預知慢型應激抑鬱症大鼠模型探討了白芍總苷的神經保護作用及其機理。實驗結果表明白芍總苷能明顯增加未預知慢性應激抑鬱模型大鼠海馬 CA3 區錐體細胞數，表現出神經保護作用。白芍總苷的神經保護作用被證實與白芍總苷抑制下丘腦-垂體-腎上腺軸活性，抑制海馬氧化應激以及增加神經營養因數如腦源性神經營養因數和神經營養素-3 表達相

關。

最後，應用一種抑鬱症體外模型（皮質酮所致 PC12 細胞損傷模型）研究了白芍總苷的神經保護作用及其機理。實驗結果表明白芍總苷對皮質酮所致的 PC12 細胞損傷有明顯的保護作用。白芍總苷的體外神經保護作用與白芍總苷抑制氧化應激以及增加神經營養因數如腦源性神經營養因數，神經生長因數和神經營養素-3 表達相關。

綜上所述，白芍總苷具有顯著的抗實驗性抑鬱症作用。白芍總苷抗抑鬱作用機制可能與白芍總苷抑制下丘腦-垂體-腎上腺軸活性，抑制氧化應激，增加神經營養因數表達，進而起到神經保護作用相關。

Publications based on the work in this thesis

Mao QQ, Ip SP, Tsai SH, Che CT (2008) Antidepressant-like effect of peony glycosides in mice. *Journal of Ethnopharmacology* 119: 272-275.

Mao QQ, Ip SP, Ko KM, Tsai SH, Che CT (2009) Peony glycosides produce antidepressant-like action in mice exposed to chronic unpredictable mild stress: effects on hypothalamic-pituitary-adrenal function and brain-derived neurotrophic factor. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 33: 1211–1216.

Mao QQ, Xian YF, Ip SP, Tsai SH, Che CT (2010) Long-term treatment with peony glycosides reverses chronic unpredictable mild stress-induced depressive-like behavior via increasing expression of neurotrophins in rat brain. *Behavioural Brain Research* 210: 171-177.

Mao QQ, Ip SP, Ko KM, Tsai SH, Zhao M, Che CT (2009) Peony glycosides protect against corticosterone-induced neurotoxicity in PC12 Cells. *Cellular and Molecular Neurobiology* 29: 643–647.

Mao QQ, Xian YF, Ip SP, Tsai SH, Che CT. Protective effects of peony glycosides against corticosterone-induced cell death in PC12 cells via antioxidant mechanisms. *Journal of Ethnopharmacology* (submitted for publication).

Mao QQ, Ip SP, Tsai SH, Che CT (2008) Antidepressant-like effect of total glycosides of peony in mice. Presented at: *7th Joint Meeting of AFERP, ASP, GA, PSE & SIF* August 3-8, Athens, Greece.

Mao QQ, Ip SP, Tsai SH, Che CT (2009) Antidepressant-like effect of the glycoside-rich extract of peony in a rat model of depression. Presented at: *50th Anniversary Meeting of the American Society of Pharmacognosy* June 27-July 1, Honolulu, Hawaii, USA.

Mao QQ, Ip SP, Tsai SH, Che CT (2009) Exploring antidepressive mechanism of the glycoside-rich extract of Radix Paeonia Alba in rats. Presented at: T*he 1st Macao Forum and The 1st PhD Student Forum on Chinese Medicine Sciences* November 12-14, Macao SAR, China.

Acknowledgement

I would like to express my sincere gratitude first and foremost to Professor Che Chun-Tao, for his kind guidance and support during the period of my research work. My heartfelt gratitude also goes to Dr. Ip Siu-Po for his constant support in this project and helpful instruction.

I am also sincerely grateful to Professor Ko Kam-Ming (Department of Biochemistry, The Hong Kong University of Science & Technology) for his assistance in these project. Special thanks also go to Dr. Tjong Yung-Wui and Ms. Xian Yan-Fang for their assistance in animal experiments. The helpful assistance in cell culture offered by Dr. Chen Meng-Li and Dr. Liang Jing are also deeply appreciated. To Dr. Tsai Sam-Hip, his assistance in chemical analysis is appreciated. Thanks also go to Professor Lin Zhi-Xiu, Dr. Zhao Ming, Dr. Yang Jia-Ming, Mr. Lan Ling-Ling, Ms. Hong Si-Jia, Mr. He Yi, Ms Zong Yu-Ying and other people in the School of Chinese Medicine who have helped me a lot.

I owe my last and deepest gratitude to my beloved mother and father for their selfless loving, constant support and encouragement.

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ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
AVP	Arginine vasopressin
BDNF	Brain-derived neurotrophic factor
Ca^{2+}	Calcium ion
CA	Cornu ammonis
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CHM	Chinese herbal medicine
CRH	Corticotropin-releasing hormone
CUMS	Chronic unpredictable mild stress
DCFH-DA	2',7'-Dichlorofluorescin diacetate
DMRIs	Dual monoamine reuptake inhibitors
DG	Dentate gyrus
ELISA	Enzyme-linked immunosorbent assay
GR	Glucocorticoid receptor
GSH	Reduced glutathione
HPA axis	Hypothalamic-pituitary-adrenal axis
IL-1	Interleukin-1
MAOIs	Monoamine oxidase inhibitors
MDA	Malondialdehyde

MR	Mineralocorticoid receptor
NaSSAs	Noradrenergic and specific serotonergic antidepressants
NDRIs	Norepinephrine-dopamine reuptake inhibitors
NGF	Nerve growth factor
NO	Nitric oxide
NOS	Nitric oxide synthase
NRIs	Norepinephrine reuptake inhibitors
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
PC12 cell	Rat adrenal pheochromocytoma cell
PGE2	Prostaglandin E2
ROS	Reactive oxygen species
SNRIs	Serotonin-norepinephrine reuptake inhibitors
SOD	Superoxide dismutase
SSRIs	Selective serotonin reuptake inhibitors
TCAs	Tricyclic antidepressants
TGP	Total glycosides of peony
Trk	Tyrosine protein kinase

Chapter 1 Introduction

1.1 Characteristics and prevalence of depression

Depression is a commonly-occurring, debilitating, and life-threatening psychiatric disorder. Depressive disorders are characterised by depressed mood and/or loss of interest or pleasure in nearly all activities in the presence of other symptoms such as loss of appetite, fatigue and lack of energy, sleep disturbance, restlessness or irritability, feelings of worthlessness or inappropriate guilt, difficulty in thinking, concentrating or making decisions and thoughts of death or suicide or attempts at suicide (Diagnostic and Statistical Manual IV, 2000; Linde et al., 2003; Candy et al., 2008).

Large epidemiological studies showed that the prevalence of depression is growing, with an estimated 10–30% of the population of the world (Paykel, 2003; Blendy, 2006). The ratio of patients with depression in woman was about two to three times higher than that in men (Brommelhoff et al., 2004). Suicide is a major risk in depression, with about 41% of depressive patients had suicidal ideation and 15% of them committing suicide (Mueller and Leon, 1996). Affective disorders account for considerable psychiatric morbidity

(pain and suffering), but also significant disability and consequent loss of productivity. The World Health Organization predicts that depression is now the fourth most prevalent cause of loss in human disability adjusted life years worldwide, and it will become the second by the year 2020 (Murray and Lopez, 1996).

1.2 Pathogenesis of depression

As to the psychological factors of depression, a huge body of evidence has suggested that depression arise from the complex interactions of multiple-susceptibility genes and environmental factors, which can be best conceptualized as genetic vulnerability that might be expressed in response to life events that are stressful to the vulnerable individual (Sullivan et al., 2000; Manji et al., 2001).

The pathophysiology of depression was not fully understood until the breakthrough in recent years. A serendipitous discovery of antidepressive drug showed that iproniazid enhanced monoamine transmitter function by inhibiting monoamine oxidase (Baumeister et al., 2003; Blendy, 2006; Slattery et al., 2004), while reserpine depleting monoamine transmitter would cause depression-like state (Baumeister et al., 2003). People thought

that the pathophysiology of depression involves dysfunction of monoamine neurotransmitter circuits in the central nervous system, particularly serotonin, norepinephrine and dopamine, which is called the monoaminergic hypothesis of depression (Owens, 2004). However, receent research on the pathophysiology of depression has revealed some serious gaps and limitations in the monoaminergic hypothesis. For example, drugs that target monoamines affect these neurotransmitter systems within hours of initial treatment, but antidepressant drugs exert their antidepressant action in patients after several weeks of daily treatment (Wong and Licinio, 2001). Still, the monoaminergic hypothesis does not address key issues such as the reason why antidepressants are also effective in other disorders, such as panic disorder, obsessive-compulsive disorder, and bulimia, or why some of the drugs that enhance serotonergic or noradrenergic transmission (for example, amphetamine or cocaine) are not effective in alleviating depression (Lanni et al., 2009). Despite these limitations, it is clear that the development of the monoamine hypothesis is very important in understanding depression and in the development of effective antidepressive drugs (Lanni et al., 2009). More recently, with the advance of neuroscience, it has been suggested that the hippocampus may play an important role in the neurobiological basis of depressive syndromes.

1.2.1 The role of the hippocampus in the pathophysiology of depression

The hippocampus is a major component of mammalian brain. It has recently received significant attention in mood disorders research due to its functions on the regulation of emotion and cognition. Caudal to and intimately connected with the amygdala, the hippocampus is a bilaminar grey-matter structure that forms the floor of the inferior temporal horn of the lateral ventricle and extends from the anterior margin of the ventricular horn to the splenium of the corpus callosum (Campbell and Macqueen, 2004). The hippocampus is composed of multiple subfields. Though terminology varies among authors, the terms most frequently used are the dentate gyrus (DG) and the hippocampus proper (cornu Ammonis, CA). The latter, when observed in coronal sections, can be mainly divided into three regions, termed CA1, CA2 and CA3, based on pyramidal neuron morphology (Campbell and Macqueen, 2004). The glutamatergic pyramidal and granule cells represent 90% of hippocampal neurons, and the remaining 10% are primarily γ-aminobutyric acid-producing interneurons (Campbell and Macqueen, 2004). Pyramidal and granule neurons in the hippocampus (especially in the CA3 region) have been showed to be vulnerable to psychosocial stress (McEwen, 2001), due to the hippocampus contains rich glucocorticoid receptors. Several clinical and laboratory experiments have

demonstrated that depression was closely associated with hippocampal neuronal atrophy and destruction, while chronic antidepressant treatment produced neuroprotective effect against hippocampal neuronal damage (Manji and Duman, 2001; Fuchs et al., 2004).

In clinical studies aimed to compare the volumetric differences in the hippocampus of patients suffering from depression with those in healthy controls, the results of multiple imaging analysis showed that the volume of hippocampus of these patients was significantly smaller than those of controls (Bremner et al., 2000; Colla et al., 2007; Frodl et al., 2007; Lange and Irle, 2004; MacQueen et al., 2003; Neumeister et al., 2005; Saylam et al., 2006). Additionally, MacQueen et al. (2003) and Saylam et al. (2006) have shown that the reduced volume of hippocampus in depression had a positive correlation with the disease duration. It has also been reported that the reduced volume of hippocampus in depression had a positive correlation with executive dysfunction (Frodl et al., 2007), but not with patient's age (Sheline et al., 1999).

Lucassen et al. (2001) measured apoptosis in postmortem hippocampal tissues by using in situ DNA end-labeling. The results showed that the rate of apoptosis in the DG, CA1 and CA3 regions of the hippocampus in depressive as well as in steroid-treated patients was

slightly increased. Whether this small cell loss accounts for clinical consequences observed in depressive patients remains to be clarifed. In another postmortem study, Stockmeier and colleagues (2004) found that hippocampal sections from depressive patients shrank are more than that of control subjects after histologic processing. However, the packing density of hippocampal cells was significantly increased and pyramidal neuron soma size was significantly decreased in depressive subjects as compared to control subjects.

Animal studies have revealed similar results to the clinical trials and postmortem studies. Thus Fuchs's group (Czéh et al., 2001; van der Hart et al., 2002) reported that there was a significant decrease in the volume of hippocampus in chronic stress-treated tree shrews, which is an animal model with high validity for depression (Fuchs, 2005), as compared to non-stressed control. Chronic treatment with antidepressants (such as clomipramine and tianeptine) significant prevented chronic stress-induced alterations in the volume of hippocampus (Czéh et al., 2001; van der Hart et al., 2002). It has also been reported that chronic stress-induced depression model rats showed neuronal atrophy and destruction in the hippocampal CA3 region, as indicated by significant decreases in the number of pyramidal neurons and total dendritic length, while chronic antidepressant treatment

protected hippocampus from damage induced by chronic stress (Magariños and McEwen, 1995; Magariños et al., 1999; Zhang et al., 2003; Takuma et al., 2007).

1.2.2 The mechanisms of hippocampal neuronal damage in depression

To date, the mechanisms of hippocampal neuronal damage in depression still remains to be uncovered. However, several lines of evidence have supported the involvement of hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis, and extracellular glutamate accumulation, increased production of nitric oxide (NO), increased oxidative stress and decreased neurotrophins contents (Zhang, 2001; Drzyzga et al., 2009). These factors have also been demonstrated to be involved in the pathophysiology of depression (Zhang, 2001; Drzyzga et al., 2009).

1.2.2.1 Hyperactivation of the HPA axis

A prominent mechanism of hippocampal neuronal damage in depression is hyperactivation of the HPA axis, which is a complex set of direct influences and feedback interactions among the hypothalamus, the pituitary gland, and the adrenal gland. It has been shown

that normal activation of the HPA axis results in the production of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) in the paraventricular nucleus of the hypothalamus (Sandi, 2004; Pariante and Lightman; 2006). Both peptides are released into the bloodstream, and they stimulate the production and secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH then stimulates the synthesis and release of glucocorticoids (cortisol in humans and corticosterone in rodents) from the adrenal gland (Figure 1.1). The activity of the HPA axis is controlled by the hippocampus. There are two distinct intracellular receptor subtypes referred to as the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) in the hippocampus (Reul and de Kloet, 1985). Glucorticoids, by potently regulating these two hippocampal glucocorticoid receptors, exert powerful feedback effects on the HPA axis. Levels of glucocorticoids that are seen under normal physiological circumstances seem to enhance hippocampal inhibition of HPA activity and also enhance hippocampal function in general and thereby promote certain cognitive abilities (Nestler et al., 2002). However, sustained elevations of glucocorticoids induced by the hyperactivation of the HPA axis, seen in depressive disorders and prolonged and severe stress-treated animals (Pan et al., 2006; Aihara et al., 2007; Li et al., 2007b; Watson and Mackin, 2007), could decrease hippocampal glucocorticoid receptors expression and damage hippocampal neurons,

particularly the CA3 pyramidal neurons (Magariños and McEwen. 1995; Sapolsky, 2000; Murray et al., 2008). Regardless of the nature of the damage, it would be expected that a reduction in the inhibitory control on the HPA axis would further increase circulating glucocorticoid levels and subsequent hippocampal damage (Nestler et al., 2002).

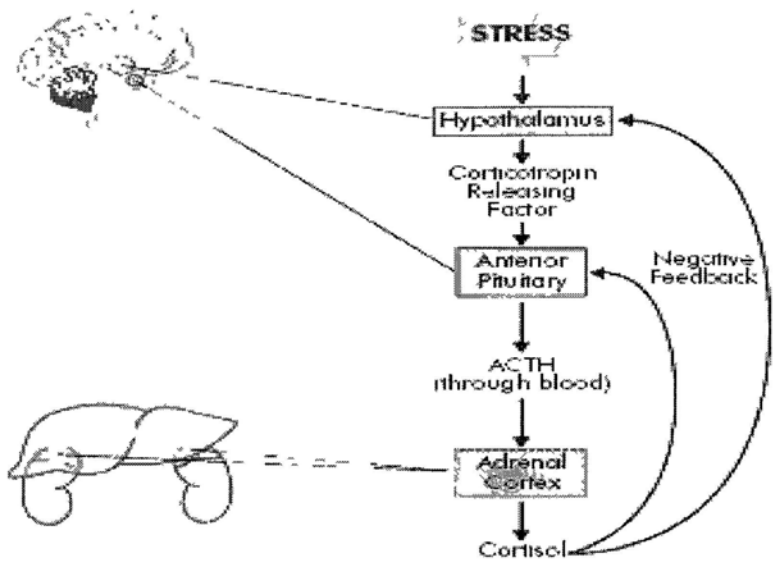


Figure 1.1 Basal activity of the HPA axis (Watson and Mackin, 2007). Normal activation of the HPA axis results in the production of CRH in the paraventricular nucleus of the hypothalamus. CRH is then released into the bloodstream, and stimulates the production and secretion of ACTH from the anterior pituitary. ACTH then stimulates the synthesis and release of glucocorticoids (cortisol in humans and corticosterone in rodents) from the adrenal gland. Glucocorticoids also play a negative feedback effect on the hypothalamus to release of CRH and anterior pituitary to release of ACTH.

1.2.2.2 Extracellular glutamate accumulation

Glutamate, one of the major endogenous excitatory neurotransmitters in the nervous system, is synthesized from glutamine by the enzyme glutaminase (Figure 1.2). Two primary glutamate receptors have been pharmacologically classified as ionotropic (N-methyl-daspartate [NMDA], AMPA, and kainate receptors) and metabotropic (G-protein coupled receptors) (Gasic and Hollmann, 1992). A physiological level glutamate is necessary for many neurological functions, including cognition, memory, movement, and sensation (Gasic and Hollmann, 1992). However, in a variety of pathologic conditions, excessive concentration of extracellular glutamate would be released and accumulated which could eventually lead to neuronal injury or death (Coyle and Puttfarcken, 1993; Obrenovitch, 1999). Interestingly, several studies have also demonstrated that overactivation of the glutamatergic system is involved in the pathogenesis of depression (Palucha and Pilc, 2002; Molina-Hernández et al., 2008; Hashimoto, 2009). It has been shown that plasma or seurm levels of glutamate in patients suffering from depression were significantly higher than those in a control group (Kim et al., 1982; Mauri et al., 1998; Mitani et al., 2006). Moreover, Mitani et al. (2006) reported that there was a positive correlation between plasma glutamate levels and severity

of depressive symptoms in depressive patients. Preclinical studies showed that high accumulation of glutamate can induce depression-like behaviours (Rada et al., 2003), while several glutamate antagonists produce antidepressant-like effects (Molina-Hernández et al., 2008). Brain gutamate level was significantly increased in a rat model of depression, and was normalized by treatment with antidepressant drugs (Hu et al., 2006).

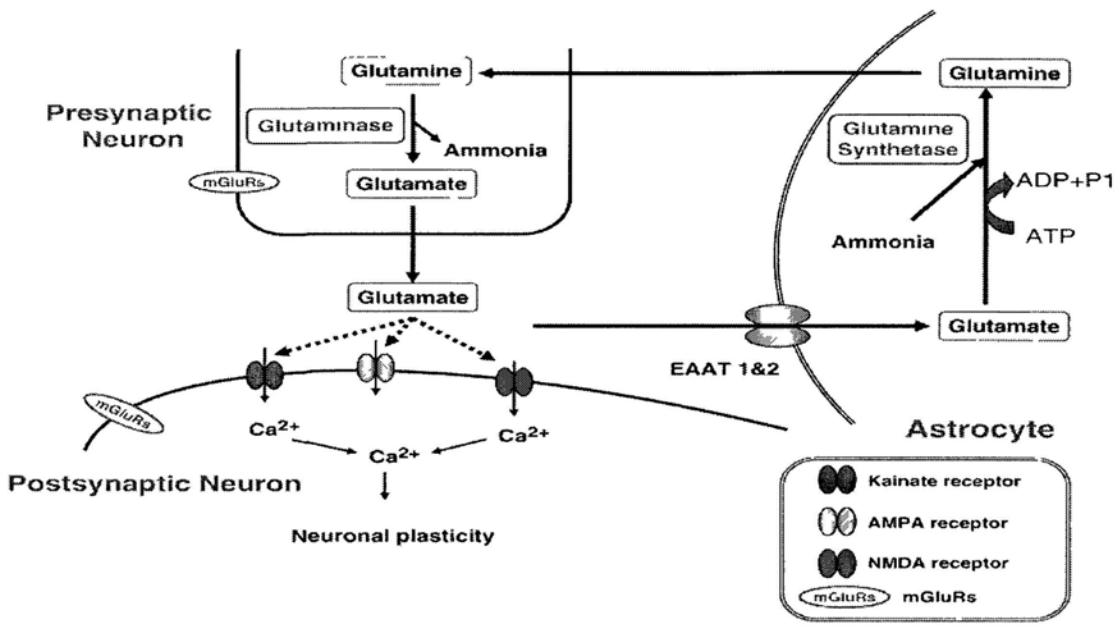


Figure 1.2 Glutamate synthesis and excitotoxicity pathways (Hashimoto, 2009). Glutamate is one of the principal excitatory neurotransmitters and synthesized from glutamine by the enzyme glutaminase. Glutamate and its ts interactions with specific neurocyte membrane receptors are responsible for many neurological functions. In a variety of pathologic conditions, excessive concentration of extracellular glutamate would overactivate the glutamate receptors, and then ncrease intracellular Ca^{2+} concentration. High concentration of intracellular Ca^{2+} triggers a cascade of membrane, cytoplasmic, and nuclear events leading to neurotoxicity.

Glutamate excitotoxicity is thought to be a consequence of intracellular calcium ion (Ca^{2+})-loading. Under normal physiological conditions, intracellular cytosolic free Ca^{2+}, regulated by plasma membrane Ca^{2+} transporters, is maintained at very low concentrations relative to free extracellular calcium (Luoni et al., 2000). However, overactivation of glutamate receptor stimulated by excessive glutamate increases intracellular Ca^{2+} concentration by directly opening ion channels and secondarily affecting Ca^{2+} homeostatic mechanisms (Mark et al., 2001) (Figure 1.2). High concentration of intracellular Ca^{2+} triggers a cascade of membrane, cytoplasmic, and nuclear events leading to neurotoxicity (Humar et al., 2009). Indeed, it has also been demonstrated that abnormal intracellular Ca^{2+} concentration is involved in the pathophysiology of stress-related behavior and depression (Zhong et al., 2006; Zhou et al., 2009).

1.2.2.3 Increased production of nitric oxide (NO)

Nitric oxide (NO) was first identified in the 1980s as an endothelium-derived relaxing factor in the cardiovascular system (Zhang et al., 2005). Later studies suggested that NO plays an important role in the central nervous system under both physiologic and pathologic conditions (Law et al., 2001). NO is synthesized from l-arginine by nitric

oxide synthase (NOS) using NADPH and molecular oxygen. To date, three isoforms of NOS, neuronal NOS, endothelial NOS, and inducible NOS, have been identified (Paakkari and Lindsberg, 1995). A biphsic effect of NO has been observed. Low levels and very early synthesis of NO by endothelial NOS have a neuroprotective role, while high levels and more sustained production of NO by inducible NOS or neuronal NOS lead to a neurotoxic effect (Ca´rdenas et al., 2005). It has been reported that increased and sustained NO levels, seen in depressive disorders and prolonged and severe stress-treated animals (Suzuki et al., 2001; Fu et al., 2005; Qin et al., 2005; Li et al., 2007a), could lead to a consecutive formation of superoxide that react with NO to form peroxynitrite, which can further react with other compounds to produce more toxic peroxide products, and then cause DNA damage (Beckman and Koppenol, 1996; Hong et al., 2004; Zhang et al., 2005). Moreover, NO and peroxynitrite-mediated DNA damage could induce overactivation of the poly(ADP-ribose) polymerase-1 (PARP-1) pathway which is a key pathway leading to cell death (Yu et al., 2003) (Figure 1.3).

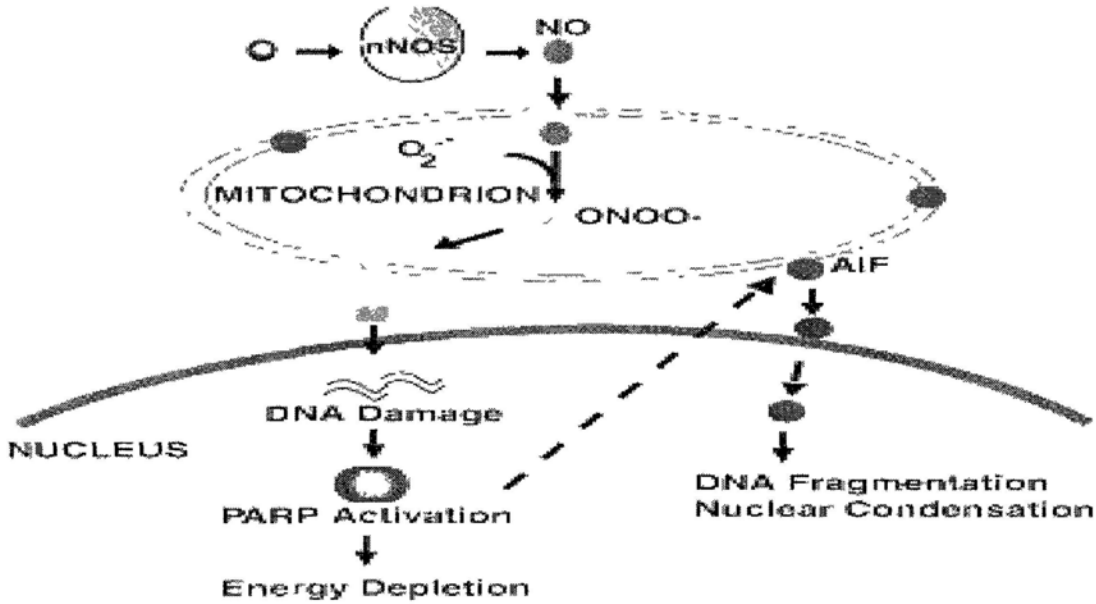


Figure 1.3 Model of NO-mediated excitotoxicity (Zhang et al., 2005). NO is synthesized from l-arginine by NOS using NADPH and molecular oxygen. In a variety of pathologic conditions, increased and sustained NO levels can lead to a consecutive formation of superoxide that react with NO to form peroxynitrite, which can further react with other compounds to produce more toxic peroxide products, and then cause DNA damage. Moreover, NO and peroxynitrite-mediated DNA damage could induce overactivation of the PARP-1 pathway which is a key pathway leading to cell death.

1.2.2.4 Increased oxidative stress

Under normal conditions, a dynamic balance exists between the production of reactive oxygen species (ROS) and the enzymatic and non-enzymatic anti-oxidant defense systems of the cell (Geier et al., 2009). ROS, mainly includes superoxide anion, hydrogen peroxide and hydroxyl radical, is produced when the cells generate energy by reducing molecular oxygen to water (Wakamatsu et al., 2008). However, endogenous antioxidant

defense neutralizes the ROS within the cells (Figure 1.4). Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase are the primary enzymes involved in the elimination of ROS (Geier et al., 2009). SOD scavenges superoxide anion to more stable hydrogen peroxide. CAT and glutathione peroxidase are two of the most important scavengers of hydrogen peroxide which convert it into water (Griendling and Ushio-Fukai, 2000). Additionally, glutathione (GSH), the most abundant endogenous antioxidant, is also involved in the antioxidant defense system (Mueller et al., 2001). Oxidative stress occurs when ROS levels exceed the antioxidant capacity of a cell in a variety of pathologic conditions. Excessive ROS are highly toxic and react with lipids, proteins and nucleic acids, and lead to cell death (Niebrój-Dobosz et al., 2004; Zhao et al., 2008) (Figure 1.4).

The brain and nervous system are particularly vulnerable to oxidative stress because of their limited antioxidant capacity, high oxygen utilization and the high content of unsaturated fatty acids (that are more liable to peroxidation) (Geier et al., 2009). The brain makes up about 2% of body mass but consumes 20% of metabolic oxygen. The vast majority of energy is used by the neurons (Geier et al., 2009; Ribeiro et al., 2009). Interestingly, several studies have also shown that there was a significant increase in the oxidative stress in depression (Lukash et al., 2002; Lucca et al., 2009), suggesting that

increased hippocampal oxidative stress may contribute to hippocampal neuronal death in depression.

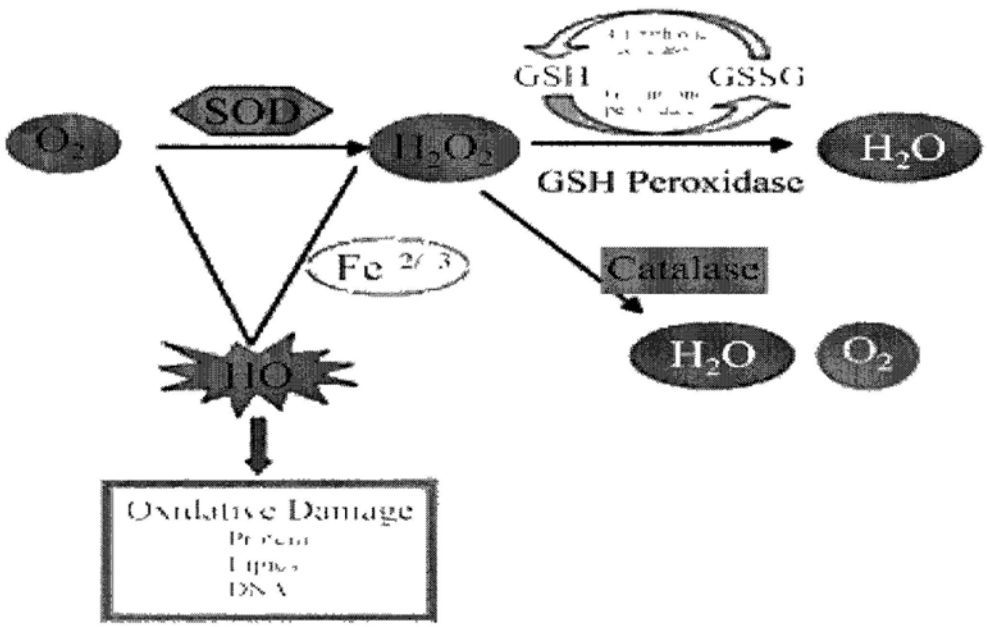


Figure 1.4 An balance between the production of ROS and anti-oxidant defense systems in cells (Wakamatsu et al., 2008). Cells generate energy by reducing molecular oxygen to water. During this process, ROS may be produced as an unavoidable by-product of mitochondrial respiration and can cause cell injury. The toxic effect of ROS can be eliminated by antioxidative enzymes such as SOD, catalase and GSH peroxidase, and antioxidants such as GSH.

1.2.2.5 Decreased neurotrophins contents

Four neurotrophins have been characterized in mammals, reptiles, and amphibians: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4, NT-4/5) (Huang et al., 2001; Chan et al., 2003). Neurotrophins exert their function by interacting with the high-affinity tyrosine protein kinase (Trk) receptors (TrkA, TrkB and TrkC). Each of these neurotrophins specifically binds to its cognate Trk receptor. NGF binds to TrkA, while BDNF and NT-4 interact with TrkB. Besides, NT-3 primarily binds to TrkC though it can also interact with TrkA or TrkB. In addition, all neurotrophins can bind with the low-affinity p75 neurotrophin receptor that may be important in enhancing the specificity of the Trk receptors (Abe, 2001; Chan et al., 2003) (Figure 1.5). The biological effects of neurotrophins are mediated either by Trk receptor signalling alone or by the co-activation of Trk and low-affinity p75 neurotrophin receptors, leading to the activation of different types of protein kinases and a cascade of intracellular events (Huang et al., 2001; Chan et al., 2003), which eventually induce the development, survival, plasticity, and differentiation of neurons (Huang et al., 2001; Blum and Konnerth, 2005). On the other hand, several recent studies have demonstrated that decreased neurotrophins contents are involved in the

pathophysiology of stress-related behavior and depression (Nestler et al., 2002; Angelucci et al., 2003; von Richthofen et al., 2003; Lang et al., 2004a; Dwivedi et al., 2005; Huston et al., 2009), and the reversal of this deficiency during antidepressant treatments may contribute to the improvement of depressive symptoms (Hellweg 2002; Angelucci et al., 2003; Xu et al., 2003; Zheng et al., 2006; Chen et al., 2007; Li et al., 2007b; Song et al., 2009), which is known as the neurotrophic hypothesis of depression (Nestler et al., 2002). These data strongly suggest the importance of neurotrophins in hippocampal neuronal damage in depression.

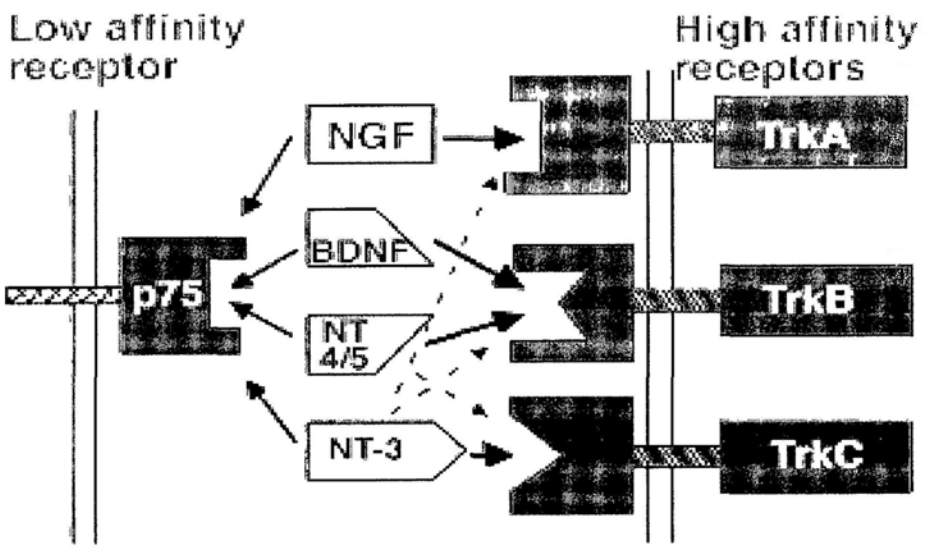


Figure 1.5 Different receptor types of neurotrophins (Gilbert SF, 2006). Each of neurotrophins specifically binds to its cognate Trk receptor. NGF binds to TrkA, while BDNF and NT-4 interact with TrkB. Besides, NT-3 primarily binds to TrkC though it can also interact with TrkA or TrkB. In addition, all neurotrophins can bind with the low-affinity p75 neurotrophin receptor that may be important in enhancing the specificity of the Trk receptors

1.3 Stress induced animal models of depression

Animal model research has an important contribution to understand the pathological mechanisms of human psychiatric disorders. It is well known that animal models of depression can provide crucial information on the whole range of function which is not accessible in humans and potentially allow us to identify novel targets for antidepressant drug development. The problem in all animal models is to define the clear criteria that allow the validity of the model to be ascertained. According to Geyer and Markou (1995), four main criteria need to be considered in order to determine the validity of animal models of behavioural disorders: (1) face validity: the phenomenological similarity between the behaviour exhibited by the animal model and the specific symptoms of the human condition; (2) etiological validity: this concept of etiological validity is closely related to the causes of the disorder in humans.; (3) predictive validity: the ability to predict changes in the human subject based upon changes in the model and (4) construct validity: construct validity is closely related to the pathology and symptomatology of the disorder, and the accuracy with which changes in the model organism reflects that in the human.

Over the course of the last 50 years, there are over 20 depressive animal models, such as neurochemical models, natural genetic models, olfactory bulbectomy model, operant response models, stress models and so on (Jesberger and Richardson, 1984; Bourin et al., 2000; McArthur and Borsini, 2006). Among these models of depression, stress is mostly used to induce depression (Willner, 1997 and 2005) because stress is a crucial psychological factor involved in the onset and relapse of depression (Bidzinska, 1984; Sheline, 2000; Lee et al., 2002). The learned helplessness model, the behavioral despair model and the chronic unpredictable mild stress model are three of them that have been extensively studied.

1.3.1 Learned helplessness model

The learned helplessness model was proposed by Seligman and colleagues (Overmier and Seligman, 1967; Seligman and Beagly, 1975; Seligman et al., 1975; Maier and Seligman, 1976) during the 1960–1970s. A presumed state of depression is induced in animals by exposing them to aversive stimuli like shock under circumstances in which they cannot control or predict the onset or duration of the stimuli. This procedure results in long-lasting deficits in the motivation and ability to escape in subsequent trials where

escape is possible, and show behavioural alterations such as vocalizations and passivity (Overmier and Seligman, 1967; Seligman and Beagly, 1975; Seligman et al., 1975; Ferguson et al., 2000; Zhang et al., 2002); as well as alterations in sleep–wake patterns (Adrien et al., 1991). Pharmacological treatment with antidepressants reversed these behavioural changes (Ferguson et al., 2000; Zhang et al., 2002).

1.3.2 Behavioral despair model

Behavioral despair model was proposed as a simpler variation of the learned helplessness model. So far, the behavioral despair model includes the forced swim test (Porsolt et al., 1977) and the tail suspension test (Steru et al., 1985), and has been the most widely used test to screen novel antidepressant compounds. In the forced swim test (Porsolt et al., 1977; Li et al., 2003), a mouse or rat is placed in a cylinder (diameter: 14 and 21 cm for mice and rats, respectively) containing water (10 cm and 25 cm in height for mice and rats, respectively), maintained at 25 ± 1 °C. After a period of time, the animal becomes immobile, or floats passively (Porsolt et al., 1977). For tail suspension test, a mouse is suspended by the tail approximately 5 cm above the floor. The subject immediately engages in struggling or escape-like behaviors, which gradually abates to immobility

(Steru et al, 1985). The behavioral phenomena observed in both forced swim and tail suspension test apear to be resulted from the exposure to inescapable aversive situations, and an animal alternates between periods of vigorous activity (searching behavior) and immobility (waiting behavior) (Porsolt et al., 1977; Steru et al., 1985). Multiple classes of antidepressant drugs have been shown to decrease immobility in the forced swim and tail suspension tests following acute or subchronic treatment (Porsolt et al., 1977; Steru et al., 1985).

While in some reports, the specificity of behavioral despair model for antidepressants has been questioned because a large number of non-antidepressive drugs also reduce immobility. False positive results have been reported for agents that stimulate locomotor activity (Porsolt et al., 1978 and 1981). On the other hand, it has been reported that the effects of stimulants such as caffeine or amphetamine can be excluded by the open field test, in which these agents show marked increase in locomotor activity (Porsolt et al., 1978). The open field test was first described by Hall (1934) in the study of emotionality in rats. Until now, the open field test has been one of the most popular procedures for studying animal psychology (Calabrese, 2008). Therefore, the combined use of behavioral despair test and open field test is a reliable method for screening potential

antidepressants, and this has been confirmed in a number of recent studies (Mora et al., 2006; Kaster et al., 2007; Binfaré et al., 2009; Carbajal et al., 2009).

1.3.2 Chronic unpredictable mild stress model

Humans are more likely to be subjected to periods of stress during their lifetime. Some people are less resilient to these stresses, and can be vulnerable to mild but prolonged stress (Kessler, 1997; Hammen, 2005; McArthur and Borsini, 2006). Therefore, a stress model of depression seeking to simulate this environmental condition was initially proposed and developed by Katz and his colleagues (Katz and Schmaltz, 1980 and Katz et al., 1981) who treated rats to various harsh stressors, such as electrical shock, immersion in cold water, reversal of light/dark cycle, fast, isolation, tail pinch, being shaken, moved from cage to cage over a period of 3 weeks. Some animals exposed to this regime may die and the survived animals show signs of locomotor inhibition and depression (Katz and Schmaltz, 1980 and Katz et al., 1981).

The chronic stress model was further improved by Willner et al. (1984) whereby rodents are subjected to similar but milder and longer (5-10 weeks) stressors than those used by

Katz, and this model is known as chronic unpredictable mild stress model. Following such exposure, animals have been reported to exhibit anhedonia-like behaviour, as indicated by a reduction in responsiveness to pleasurable stimuli, measured by a decrease in their consumption of 1% or 2% sucrose solution or a decrease in their preference for sucrose over water (Willner, 1997 and 2005). In addition, a number of behavioural and physiological changes have been reported, including decreased sexual behaviour, decreased aggression, decreased body weight, alterations in locomotor activity, adrenal hypertrophy, corticosterone hypersecretion and decreased REM sleep (D'Aquila et al., 1994; Papp et al., 1994; Norman and McGrath, 2000). These behavioural symptoms induced by chronic unpredictable mild stress are often observed in patients suffering from major depression and can be reversed by chronic treatment with antidepressants, indicating the model has good predictive validity (D'Aquila et al., 1994; Papp et al., 1994; Li et al., 2007b; Zhao et al., 2008). In fact, chronic unpredictable mild stress model of depression has been generally accepted as the most promising and valuable depressive model in animals (Willner, 1997 and 2005).

1.4 Drugs for treating depression

Prior to the late 1950s, depression was treated primarily with electroconvulsive therapy and psychotherapy. The advent of effective antidepressants changed this situation and drug therapy has become the main modality for treating depression (Hollister, 1978). The number of drugs available for treating depression has been growing rapidly during the past 60 years. Several types of antidepressant medications were identified according to their different mechanism of action: tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), selective serotonin reuptake inhibitors (SSRIs), norepinephrine reuptake inhibitors (NRIs), dual monoamine reuptake inhibitors (DMRIs) including serotonin-norepinephrine reuptake inhibitors (SNRIs) and norepinephrine-dopamine reuptake inhibitors (NDRIs), noradrenergic and specific serotonergic antidepressants (NaSSAs), and herbal medicines.

1.4.1 TCAs

TCAs are one of the oldest classes of antidepressant drugs. Since the early 1960s, TCAs represented the major pharmacological treatment for depression (Pacher and Kecskemeti,

2004). They work by blocking the reuptake of serotonin and norepinephrine and producing long-term increases in sensitivity in these receptors. They also produce a range of potentially serious side effects including drowsiness, dry mouth, constipation, urinary retention, blurred vision, dizziness, confusion, and sexual dysfunction (Steffens et al., 1997; Kyle et al., 1998; Roose et al., 1998). However, TCAs are still used in clinic because of their effectiveness, especially in severe cases of major depression (Anderson, 1998; Barbui et al., 2004). TCAs mainly include amitriptyline, clomipramine, desipramine, doxepin, imipramine, nortriptyline, protriptyline and trimipramine in the pharmaceutical market (Pacher and Kecskemeti, 2004; Woolf et al., 2007). The human dosage of various TCAs are shown in Table 1.1.

Table 1.1 The human dosage of TCAs and MAOIs (Hollister, 1978; Tong and Tong, 2009)

Antidepressants	Name	Molecular Formula	Human dosage (mg/d)
TCAs	Amitriptyline	$C_{20}H_{23}N$	50-250
	Clomipramine	$C_{19}H_{23}ClN_2$	100-200
	Doxepin	$C_{19}H_{21}NO$	50-200
	Desipramine	$C_{18}H_{22}N_2$	75-150
	Imipramine	$C_{19}H_{24}N_2$	100-300
	Nortriptyline	$C_{19}H_{21}N$	30-75
	Protriptyline	$C_{19}H_{21}N$	15-60
	Trimipramine	$C_{20}H_{26}N_2$	75-300
MAOIs	Dextroamphetamine	$C_9H_{13}N$	10-60
	Isocarboxazide	$C_{12}H_{13}N_3O_2$	20-50
	Phenelzine	$C_8H_{12}N_2$	15-75
	Tranylcypromine	$C_9H_{11}N$	10-30
	Moclobemide	$C_{13}H_{17}ClN_2O_2$	300-600

1.4.2 MAOIs

MAOIs are also one of the oldest classes of antidepressant drugs. The "first-generation" MAOIs mainly include dextroamphetamine, isocarboxazide, phenelzine and tranylcypromine in the pharmaceutical market (Pacher and Kecskemeti, 2004). They work by irreversibly blocking the action of monoamine oxidase enzyme, which breaks down the neurotransmitters dopamine, serotonin and norepinephrine. It has been shown that "first-generation" MAOIs are more efective than TCAs for atypical depression, as well as in persons who do not respond to other type antidepressants (McGrath et al., 1993; Stewart et al., 1997). However, because of the potential risk of life-threatening drug-food interactions (Beavis, 1998) and a higher incidence of dangerous side effects (Taavitsainen et al., 2001; Salsali et al., 2004), they are used only for patients who are refractory to other medications (Zetin et al., 2006). Moclobemide, a reversible inhibitor of monoamine oxidase type-A, is the "second-generation" MAOIs. It has been reported that moclobemide did not require dietary restrictions and have fewer adverse effects than traditional irreversible MAOIs (Yamada and Yasuhara, 2004). The human dosage of various MAOIs are shown in Table 1.1.

1.4.3 SSRIs

SSRIs are a family of antidepressants which are considered as the current standard of drug treatment for all forms of depression (Pacher and Kecskemeti, 2004). They work by preventing the reuptake of serotonin by the presynaptic neuron, thus maintaining higher levels of serotonin in the synapse. Five main SSRIs are now available in the pharmaceutical market: citalopram, fluoxetine (the first SSRIs occurred in 1988), fluvoxamine, paroxetine and sertraline (Pacher and Kecskemeti, 2004). These drugs are approximately as effective as TCAs, although some evidence suggested that TCAs might be more effective than SSRIs for severe depression (Anderson, 1998 and 2000; Barbui et al., 2004). It has also been shown that SSRIs had a much more benign and tolerated side effect profile than TCAs and, largely for this reason, have replaced TCAs as the first line therapy for depression (Pacher and Kecskemeti, 2004). However, the major problem of SSRIs is sexual dysfunction (Rosen et al., 1999). The human dosage of various SSRIs are shown in Table 1.2.

Table 1.2 The human dosage of SSRIs (Koenig amd Thase, 2009; Tong and Tong, 2009)

Name	Molecular Formula	Human dosage (mg/d)
Citalopram	$C_{20}H_{21}FN_2O$	20-60
Fluoxetine	$C_{17}H_{18}F_3NO$	20-60
Fluvoxamine	$C_{15}H_{21}F_3N_2O_2$	50-300
Paroxetine	$C_9H_{11}N$	10-30
Sertraline	$C_{17}H_{17}Cl_2N$	50-200

1.4.4 NRIs

NRIs represent a class of antidepressant drugs that inhibit the reuptake of norepinephrine by the presynaptic neuron, thus maintaining higher levels of norepinephrine in the synapse. These drugs have been shown to be approximately equivalent to TCAs and SSRIs in efficacy (Scates and Doraiswamy, 2000; Hajós et al., 2004; Papakostas et al., 2008). NRIs have a much more benign and tolerated side effect profile than TCAs (Hajós et al., 2004), while they produce more distinct adverse side effect profile than SSRIs, showing urinary and cardiovascular side effects of greater concern (Pacher and Kecskemeti, 2004). Reboxetine ($C_{19}H_{23}NO_3$) is probably the most used NRIs in the pharmaceutical market.

Doses are usually 4-10 mg/d in human (Tong and Tong, 2009).

1.4.5 DMRIs

DMRIs are a newer form of antidepressants that work by preventing the reuptake of two monoamines by the presynaptic neuron. There are two classes of dual monaoamine reuptake inhibitors: serotonin-norepinephrine reuptake inhibitors (SNRIs) and norepinephrine-dopamine reuptake inhibitors (NDRIs). SNRIs affect norepinephrine and serotonin, while NDRIs affect dopamine and norepinephrine. It has been hypothesized that dual reuptake inhibitors might have faster onset of action or are more efficactive than single monoamine reuptake inhibitors (Thase et al., 2001; Thompson, 2002), and the results were confirmed in a clinical study (Thase et al., 2001). Dual monaoamine reuptake inhibitors have more side effects (drowsiness, headache, nausea, changes in appetite, vivid dreams, and sexual malfunction) than SSRIs because they work with two monaoamines (Isaac, 2008). Five main DMRIs are now available in the pharmaceutical market: duloxetine, milnacipran, trazodone and venlafaxine (SNRIs) and bupropion (NDRIs) (Pacher and Kecskemeti, 2004). The human dosage of various DMRIs are shown in Table 1.3.

Table 1.3 The human dosage of DMRIs (Koenig amd Thase, 2009; Tong and Tong, 2009)

MDRIs	Name	Molecular Formula	Human dosage (mg/d)
SNRIs	Duloxetine	$C_{18}H_{19}NOS$	30-60
	Milnacipran	$C_{15}H_{22}N_2O$	50-100
	Trazodone	$C_{19}H_{22}ClN_5O$	50-300
	Venlafaxine	$C_{17}H_{27}NO_2$	75-375
NDRIs	Bupropion	$C_{13}H_{18}ClNO$	150-300

1.4.6 NaSSAs

NaSSAs are a new class of antidepressants. They have been proposed to act by noradrenergic autoreceptor and heteroreceptor antagonism combined with specific serotonergic antagonism. This results in increases in both noradrenergic and specific serotonergic transmission. Many clinicians consider NaSSAs as a second-line or even third-line antidepressant, they are used only when other antidepressants are tolerated or ineffective (Hartmann, 1999). NaSSAs are said to have fewer side effects than TCAs and SSRIs but they still carry side effects such as weight gain, drowsiness, and increased appetite (Kent, 2000; Schweitzer et al., 2009). Mirtazapine ($C_{17}H_{19}N_3$) is probably the

most popular NaSSAs in the pharmaceutical market (Schweitzer et al., 2009). Doses are usually 15-45 mg/d for human (Tong and Tong, 2009).

1.4.7 Herbal medicines

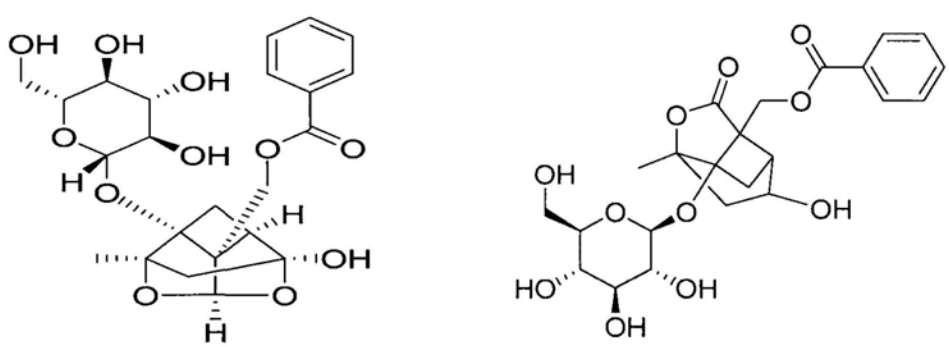
The use of herbal drugs, which are expected to show fewer side-effects, has been gaining wide popularity in the world. St. John's wort (*Hypericum perforatum*) is a herbal medicine that has been traditionally used to treat a variety of health conditions such as nerve pain, malaria, viral, and bacterial infection (National Committee of Chinese Pharmacopoeia, 2005). A number of controlled clinical studies showed that the standard extract of St. John's wort (trade name: neurostan, contains 3% hypericin content) can be used to treat mild and moderate depression, with a favourable side-effect profile (Stevinson and Ernst, 1999; Nangia et al., 2000; Bilia et al., 2002; Linde et al., 2003; Rodriguez-Landa and Contreras, 2003). However, St. John's wort is less effective than other standard antidepressants in severe depression (Hypericum Depression Trial Study Group, 2002). The mechanisms by which St. John's wort exerts its antidepressive effects remain to be elucidated because of its complexity in the chemical profile of biologically active compounds (Nahrstedt and Butterweck, 1997). Doses are usually 300-1800 mg/d

in human.

On the other hand, Chinese herbal medicine (CHM) has been used to treat psychiatric disorders in China. Xiaoyao-San (Tai Ping Hui Ming He Ji Ju Fang) is a CHM formula consist of eight components herbs, namely, Chai hu (Radix Bupleuri Chinensis), Dang gui (Radix Angelica), Bao shao (Radix Paeoniae Alba), Bai zhu (Rhizoma Atractylodis Macrocephalae), Fu ling (Rhizoma Smilacis), Sheng jiang (Rhizoma Zingiberis Recens), Bo he (Herba Menthae) and Gan cao (Radix Glycyrrhizae). A clinical study showed that Xiaoyao-San was effective against depressive neurosis (Zhang et al., 1998). Danzhi Xiaoyao-San is modified from the Xiaoyao-San; it has the addition of Dan zhi (Cortex Moutan) and Zhi zi (Fructus Gardeniae). Danzhi Xiaoyao-San has been demonstrated to have no statistical difference with reference antidepressant (maprotiline), with both being highly effective in treating depression (Luo et al., 2006). Another example of CHM formula useful for relieving depressive-like symptoms in the clinic is Jiawei Sini-San (Xie and Wang, 2005). The formula is composed of five herbs, namely, Chai hu (Radix Bupleuri Chinensis), Bao shao (Radix Paeoniae Alba), Gan cao (Radix Glycyrrhizae), Zhi shi (Fructus aurantii Immaturus) and Gui zhi (Ramulus Cinnamomi).

1.5 Pharmacological activities of total glycosides of peony (TGP)

The root of *Paeonia lactiflora* Pall. (Family: Ranunculaceae), commonly known as peony, is a well-known herbal medicine used in China, Korea and Japan for thousands of years. Total glycosides of peony (TGP) is the biologically active ingredients of peony. As reported by Zhang and Li (2002), Wang et al. (2005a) and Xu et al. (2007), TGP contained a series of glycosides including albiflorin, benzoylpeoniflorin, benzoyloxypaeoniflorin, hydroxypeoniflorin, oxypaeoniflorin, xoybenzoylpaeoniflorin and peoniflorin, etc. Peoniflorin and albiflorin (Figure 1.6), account for more than 75% of the active ingredients of TGP (Wang et al., 2005b). A number of recent investigations have demonstrated that TGP possesses anti-cancer, anti-inflammatory, analgesic, anti-oxidant, hepatoprotective, neuroprotective, renoprotective properties.



Paeoniflorin **Albiflorin**

Figure 1.6 The chemical structure of paeoniflorin and albiflorin.

1.5.1 Anti-cancer effects

More and more attentions are paid in the search of antitumor drugs to prevent and cure cancer. A clinical study showed that the combined use of TGP and chemotherapy remedy in treating non-small cell lung carcinoma produced better results than chemotherapy treatment alone (Zhan et al., 2006), suggesting an adjuvant role of TGP in cancer treatment. *In vitro* studies have focused on the effects of TGP on hepatic cancer. It has been reported that TGP had inhibitory effects on the proliferation of SMMC-27721 cell line (a human hepatoblastoma cell line) in a concentration-dependent manner. This effect was associated with the apoptotic effect of TGP (Wang et al., 2006a). Results from Wang et al. (2006B) showed that TGP concentration-dependently inhibited the proliferation of $HepG_2$ cells (a human hepatoblastoma cell line) by inducing apoptosis.

1.5.2 Anti-inflammatory and analgesic effects

TGP has been shown to process anti-inflammatory effects in acute, subacute and chronic animal models of inflammation, such as dimethylbenzene-induced ear edema in mice, carrageenan-induced paw swelling in rats and tampon-induced granuloma (Gao et al.,

2002). The mechanisms underlying the anti-inflammatory effects of TGP might be related to the inhibition of leukotriene activity, interleukin-1 (IL-1) activity, prostaglandin E2 (PGE2) content, iNOS and NO content (Li et al., 1994; Zhou and Li, 2003; Chen et al., 2008). Gao et al. (2002) also studied the analgesic effects of TGP by using writhing test. The results showed that TGP treatment markly reduced the writhing frequency induced by acetic acid in mice. Wang et al. (1988) also found that TGP treatment significantly elevated the pain threshold in the hot-plate test in mice, suggesting the analgesic effects of TGP. Rheumatoid arthritis is an inflammatory joint disease. TGP has been approved by State Food and Drug Administration of China as a drug for treating rheumatoid arthritis. Several studies reported that TGP exerted anti-inflammatory and analgesic effects in the rat model of complete Freund's adjuvant-induced adjuvant arthritis, collagen-induced arthritis and carrageenan-induced arthritis (Wang et al., 1996; Xu et al., 2007; Chang et al., 2009), which is mediated by regulating the level cyclic adenosine monophosphate (cAMP) and the Bcl-2/Bax ratio, and inhibiting the production of pro-inflammatory mediators and PGE_2 from activated synoviocytes (Jia et al., 2006; Xu et al., 2007; Chang et al., 2009).

1.5.3 Antioxidant activities

Gao et al. (1996) reported the antioxidant properties of TGP in some *in vitro* models of oxidative stress. The results showed that the half maximal inhibitory concentration (IC_{50}) of TGP on scavenging $O_2^{\cdot-}$ was 2.6 mg/L; the IC_{50} of TGP on scavenging OH· was 117.9 mg/L; the IC_{50} of TGP on inhibiting oxidative hemolysis induced by hydrogen peroxide was 1.4 mg/L. Effects of TGP on oxidative status under pathological conditions in animals have also been extensively investigated. TGP protected against carbon tetrachloride-induced acute hepatic injury, tripterygium glycosides tablet-induced acute hepatic injury, Bacillus Calmette-Guérin plus lipopolysaccharide-induced immunological hepatic injury and human albumin-induced hepatic fibrosis in animals. The antioxidant effet was attributed to the enhancement of the activities of hepatic antioxidative enzymes and the inhibition of hepatic lipid peroxidation (Wang et al., 2004; Wang et al., 2005a; Zhan et al., 2006; Zhou et al., 2007b). It has been demonstrated that TGP exerted beneficial effects in chronic hyperlipidemic rabbits by the inhibition of hepatic lipid peroxidation (Dong et al., 2003). Fang et al. (2008) reported that TGP could produce renoprotective effects on experimental diabetic rats via the enhancement in the activities of antioxidative enzymes in kidney. Yang et al. (2001) showed that TGP could improve the

learning and memory capacity in senile mice, which was mediated by the inhibition of oxidative stress in brain. Moreover, findings in recent studies revealed that the protection of TGP against local cerebral ischemia injury and complete cerebral ischemia reperfusion injury in rats were associated with the enhancement of brain antioxidant status (Wu and Zhu, 2001; Liu et al., 2004). TGP could also reverse the changes in serum antioxidant status in rats exposed to mental stress (Zhou and Hu, 2007).

1.5.4 Hepatoprotective effects

In China, peony roots have been widely used to improve the liver function of patients with hepatitis and cirrhosis. In recent years, several studies have demonstrated that TGP produced hepatoprotective effects in animal models of hepatic injury. Wang et al. (2004) reported that TGP treatment significantly reversed the changes of serum transaminases (alanine aminotransferase and aspartate aminotransferase) levels in mice exposed to Bacillus Calmette-Guérin and lipopolysaccharide. The mechanisms underlying the protective effects of TGP on Bacillus Calmette-Guérin and lipopolysaccharide-induced immunological hepatic injury might be related to the inhibition of hepatic oxidative stress and the production of pro-inflammatory mediators and NO. Zhan et al. (2006) showed

that treatment with TGP significantly decreased serum transaminases levels, and attenuated the area and extent of necrosis and reduced the infiltration of inflammatory cells in liver in carbon tetrachloride-treated mice, which might be mainly attributed to the enhancement in the activities of hepatic antioxidative enzymes and the inhibition of hepatic lipid peroxidation. Zhou et al. (2007b) also showed that TGP treatment significantly decreased serum transaminases levels in tripterygium glycosides tablet-treated mice via the inhibition of oxidative stress. Moreover, Wang et al. (2005a) reported that TGP treatment improved human albumin-induced alterations in the liver structure, alleviated lobular necrosis and significantly lowered collagen content in rats. The antifibrotic effect of TGP was mediated by the inhibition of hepatic oxidative stress and serum NO level.

1.5.5 Effects on central nervous system

Neuropharmacology of TGP has attracted much interest in the last few decades. It was shown that TGP treatment significantly inhibited the locomotor activity in mice, suggesting that TGP produced sedative effects (Wang et al., 1986). Zhang et al. (1993) reported that TGP had regulatory effects on sleep rhythm, as TGP treatment significantly

enhanced the episode duration of slow-wave sleep in normal rats, and restored the sleep parameters in insomniac rats induced by caffeine. Zhang et al. (1994) have studied the anticonvulsant action of TGP. The results showed that TGP had a dose-dependent protective effect against maximal electroshock seizure in mice and also had an antagonistic effect on strychinine-induced convulsion in both mice and rats, but it did not affect metrazol minimal threshold seizure in mice. A recent study has demonstrated that TGP could increase both learning and short-term memory ability in mice and also improved the impairments of learning and memory acquisition in scopolamine-treated mice (Ming et al., 1993). Yang et al. (2001) also showed that TGP could improve the learning and memory capacity in senile mice induced by D-galactose by using shuttle and water maze tests, which was mediated by the inhibition of oxidative stress and total monoamine oxidases activities, and increasing cholinesterase content in brain. Moreover, TGP has been shown to produce protective effect against acute cerebral ischemia in both mice and rats (Liu et al., 2001). The same research group (Liu et al., 2004) further studied the protective effect of TGP on cerebral ischemia-reperfusion injury in rats. The results showed that TGP treatment markedly reduced the change of eye colour and possessed inhibitory effects on the histopathological changes of ischemia cerebral tissues, which was mediated by the inhibition of oxidative stress in brain. He et al. (2000a and 2000b) studied the

neuroprotective effect of TGP *in vitro* model of ischemia. TGP has been shown to protect against neurotoxicity in cultured primary cortex neurons induced by hypoxia, hypoglucose, potassium chloride, NO or excitatory amino acid (He et al., 2000b). TGP also produced neuroprotective effect against potassium chloride and excitatory amino acid induced cell death in PC12 cells by decreasing intracellular Ca^{2+} concentration (He et al., 2000a).

1.5.6 Renoprotective effects

TGP has been shown to protect against gastrointestinal comprehensive immunization-induced renal injury in rats, as indicated by the significant decreases in the levels of blood creatinine and urea nitrogen, and the reversal of glomerular histopathological changes (Zhou et al., 2006). On the other hand, Wu et al. (2009) reported that streptozotocin-induced diabetic rats showed a significant increase in the rate of 24-h urinary albumin excretion and the degree of tubulointerstitial injury, while TGP treatment significantly reversed these changes, suggesting that TGP produced renoprotective effects against experimental diabetes. The mechanisms underlying the renoprotective effects of TGP on experimental diabetes might be related to the inhibition

of oxidative stress, and decreased protein expressions of intercellular adhesion molecule (ICAM)-1, interleukin-1, tumor necrosis factor -α, and 3-nitrotyrosine in kidney (Fang et al., 2008; Wu et al., 2009).

1.5.7 Other pharmacological effects

It has been reported that TGP significantly reversed the decrease in hemolysin level and strengthened the intensity of delayed type of hypersensitivity reaction in cyclophosphamide-treated mice, but also inhibited the increased intensity of delayed type of hypersensitivity reaction induced by cyclophosphamide, suggesting that TGP had bidirectional effects on immunological modulation (Zhang et al., 1990). TGP also produced bidirectional immunological regulating effects on the hypothalamic-pituitary-adrenal axis in rats (Zhou et al., 1994). Yang et al. (2002) reported that TGP could produce regulating effects on the motility of colon of Guinea pig *in vitro* via increasing the expression of substance P. Moreover, TGP has also been shown to possess anti-diabetic effects in rats (Wu et al., 2009), anti-thrombotic effects in rats (Yang et al., 2006), hypothermic effects in rats (Wang et al., 1986), protective effects on acute myocardial ischemia in dogs (Wang et al., 2006c) and regulating effects on

chronic hyperlipidemic rabbits (Dong et al., 2003).

1.6 Objectives of this study

Although antidepressant drugs, which target at the monoaminergic systems, are widely available in the pharmaceutical market, they produce remission in 30% of patients (McNally et al., 2008) and often exert adverse side-effects. Therefore, it is desirable to seek antidepressants in naturally-occurring herbs; such materials are expected to show fewer side-effects. The root of *Paeonia lactiflora* Pall. (Family: Ranunculaceae), commonly known as peony, is one of the most commonly used medicinal herbs in China, Korea and Japan. On a preliminary study in our laboratory, the antidepressant-like effect of peony extract was investigated. The results showed that intragastric administration of peony extract at the doses of 250 and 500 mg/kg for seven days significantly reduced the duration of immobility in both forced swim test and tail suspension test, with did not affect locomotor activity in the open-field test (Mao, et al. 2008). TGP is generally regarded as the biologically active components of peony. However, it remains unknown whether TGP is the active principle responsible for antidepressive effects of peony. Therefore, in order to provide preclinical data for further development into a therapeutic agent, this

study aims to

1. investigate the antidepressant-like effects of TGP on behavioural despair model and chronic unpredictable mild stress (CUMS)-induced model;
2. explore the mechanisms underlying the antidepressive action of TGP.

The first step of this study is to study the antidepressant-like effects of TGP by using behavioral despair tests, forced swim and tail suspension tests (Chapter 2). Then the action of TGP is further confirmed by a rat model of depression induced by CUMS and the behavioral changes were measured by sucrose preference, open-field and forced swim tests (Chapter 3). The third step is to investigate the antidepressive and neuroprotective mechanisms of TGP in CUMS-treated rats (Chapter 4). Finally, the neuroprotective effects of TGP against corticosterone-induced neurotoxicity in rat pheochromocytoma (PC12) cells, an *in vitro* experimental model of depression, and its possible mechanisms is investigated (Chapter 5).

Chapter 2

Antidepressant-like effects of total glycosides of peony in the forced swim test and tail suspension test

2.1 Introduction

The root of *Paeonia lactiflora* Pall. (Family: Ranunculaceae), commonly known as peony, is one of the most commonly used medicinal herbs in China, Korea and Japan. It is contained in a number of traditional formulae (for example, Jiawei Sinisan and Xiaoyao-San) for the treatment of depression-like disorders (Zhang et al., 1998; Xie and Wang, 2005). A preliminary study in our laboratory demonstrated that an ethanol extract of peony displayed antidepressive effects in mouse models of depression (Mao, et al. 2008). Glycosides such as paeoniflorin and albiflorin are known to be the biologically active ingredients of peony, and its total glycosides have been shown to possess analgesic, anti-cancer, anti-diabetic, anti-inflammatory, anti-oxidant, anti-thrombotic, hepatoprotective, immunomodulatory, neuroprotective, renoprotective effects and so on (Zhang et al., 1990; Zhou et al., 1994; Liu et al., 2001; Dong et al., 2003; Yang et al., 2006; Zheng et al., 2005; Xu et al., 2007; Wang et al., 2005a; Chang et al., 2009; Wu et al., 2009;

Zhang et al., 2009). However, information regarding the antidepressant activity of total glycosides of peony (TGP) is lacking.

The forced swim test and tail suspension test are the widely used animal models of depression for screening antidepressant activity (Porsolt et al., 1977; Steru et al., 1985). This is largely due to their ease of use, reliability across laboratories, and ability to detect a broad spectrum of antidepressants (Liang et al., 2008). Therefore, the present study aims to evaluate the antidepressant-like effects of TGP by using the forced swim test and tail suspension test in mice and rats.

2.2 Materials and methods

2.2.1 Drugs and chemical reagents

The total glycosides of peony (TGP, a light yellow-to-brown powder, purity = 55%) was supplied by Ningbo Liwah Pharmaceutical Co., Ltd. (Zhejiang, China). TGP was analyzed by high-performance liquid chromatography as previously described (Department of Health, Hong Kong SAR, 2008) and standardized to contain 30% (w/w) of

paeoniflorin and 10% (w/w) of albiflorin (Figue 2.1). HPLC analytical conditions were as follows: a Waters Nova-Pak C18 HPLC column (4.6 x 250 mm) was used for the separation. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B). Separation was achieved by a linear gradient elution from 0% to 20% solvent B over 60 min at a flow rate of 1.0 ml/min. The eluate was monitored by a diode array detector at wavelength of 230.8 nm. A voucher sample (TGP071024) has been deposited in the School of Chinese Medicine, the Chinese University of Hong Kong. Clomipramine, purchased from Beijing Novartis Pharmaceutical Co., Ltd. (Beijing, China), was used as positive control. All other reagents and solvents used in the study were of analytical grade.

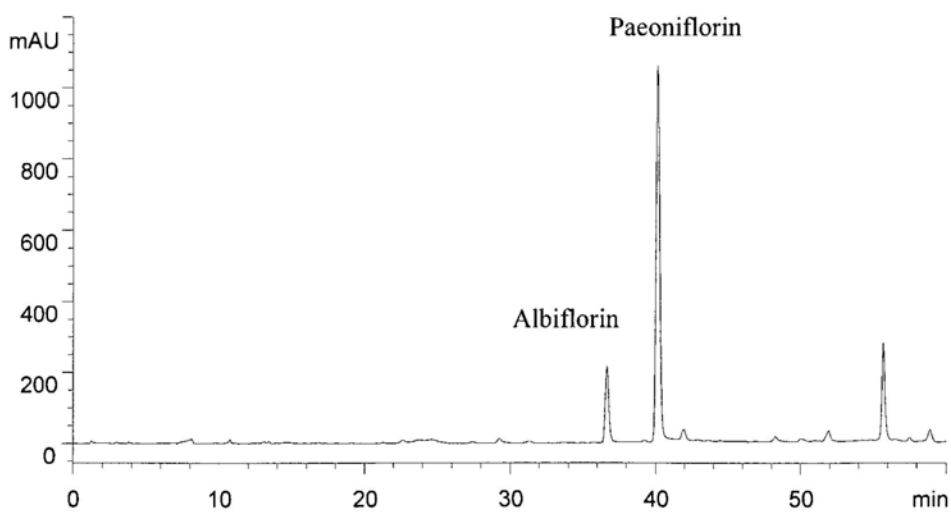


Figure 2.1 High-performance liquid chromatography of TGP.

2.2.2 Animals

Male ICR mice weighing 20-25 g and male Sprague-Dawley rats weighing 200-220 g were obtained from the Laboratory Animal Services Center, The Chinese University of Hong Kong, Hong Kong. Animals were maintained on a 12-hour light/dark cycle under regulated temperature (22 ± 2 °C) and humidity (50 ± 10 %) and fed with standard diet and water *ad libitum*. They were allowed to acclimatize for 3 days before use for experiments. The experiments on animals have been approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong and conformed to the guidelines of the "Principles of Laboratory Animal Care" (NIH publication No.80-23, revised 1996). Effort was made to minimize the number and suffering of the animals.

2.2.3. Drug administration

TGP and clomipramine were dissolved in physiological saline. All dosages were expressed as milligrams per kilogram body weight of the respective drugs. Mice or rats (n=10/group) were treated intragastrically with physiological saline, TGP (40, 80 and 160 mg/kg) and reference compounds (clomipramine 20 mg/kg) in a volume of 10 ml/kg once

daily at 9:30 to 10:30 a.m for seven days. In the clinical practice, TGP is usually prescribed at a daily dose of 480 mg. When this human dose was converted into an animal dose (a person of 60 kg, and a conversion factor of 10 between human and mouse or rat), it was equivalent to the middle dose (80 mg/kg) used in this study. The tests were conducted 60 min after the last dose.

2.2.4 Forced swim test in mice

The test was carried out according to the method described by Porsolt et al. (1977) with modifications. Briefly, mice were forced to swim in a transparent glass vessel (14 cm in diameter, 25 cm high) filled with 10 cm of water at 24–26 °C. The total duration of immobility (seconds) was measured during the last 4 min of a single 6-min test session. Mice were considered immobile when they made no attempt to escape except the movements necessary to keep their heads above the water.

2.2.5 Forced swim test in rats

The test was carried out according to a modification of the procedure described by Porsolt et al. (1977), as suggested by Li et al. (2003a) for rats. Briefly, rats were placed individually in a clear glass cylinder (diameter 21 cm, height 50 cm) filled with 25 cm of water at 24–26 °C for 15 min. The rats were then dried and returned to their home cages. On the following day, rats were re-exposed to the forced swimming for 5 min. The total duration of immobility (seconds) was quantified during a test period of 5 min. Rats were considered immobile when they made no attempts to escape except the movements necessary to keep their heads above the water.

2.2.6 Tail suspension test in mice

Tail suspension test was performed based on the method of Steru et al. (1985). Briefly, mice were suspended 5 cm above the floor by means of an adhesive tape, placed approximately 1 cm from the tip of the tail. The total duration of immobility (seconds) was quantified during a test period of 6 min. Mice were considered immobile when they were completely motionless.

2.2.7 Open-field test in mice

The ambulatory behavior was assessed in an open-field test as described previously (Herrera-Ruiz et al., 2006). The open-field apparatus consisted of a square wooden arena (30 cm × 30 cm × 15 cm) with black surface covering the inside walls. The floor of the wooden arena was divided equally into 25 squares marked by black lines. In the test, each mouse was placed individually into the center of the arena and allowed to explore freely. The number of crossings (squares crossed with all paws) and rearings (rising of the front paws) were recorded during a test period of 3 min. This apparatus was cleaned with a detergent and dried after occupancy by each mouse.

2.2.8 Open-field test in rats

Open-field test was carried out as described previously (Zhao et al., 2008) with minor modifications. The open-field apparatus consisted of a square wooden arena (100 cm ×100 cm × 40 cm) with black surface covering the inside walls. The floor of the wooden arena was divided equally into 25 squares marked by black lines. In the test, rat was placed individually into the center of the arena and allowed to explore freely. The

number of crossings (squares crossed with all paws) and rearings (rising of the front paws) were recorded during a test period of 3 min. This apparatus was cleaned with a detergent and dried after occupancy by each rat.

2.2.9 Statistical analysis

Data were expressed as mean ± SEM. The GraphPad Prism software (version 4.0) was used to perform the statistics (GraphPad Software, Inc., San Diego, CA). One-way analysis of variance (ANOVA) followed by Dunnett's test was used to test the differences between the groups. Difference was considered statistically significant when the value $p < 0.05$.

2.3 Results

2.3.1 Effect of TGP on the duration of immobility in the forced swim test in mice

Figure 2.2 shows the effect of TGP treatment on the duration of immobility in the forced swim test in mice. Treating mice with TGP at the doses of 80 and 160 mg/kg for seven days significantly reduced the duration of immobility (30% and 43%) in the forced swim test in mice, as compared to the control group. The classical antidepressant clomipramine, also caused a significant reduction in the immobility time (44%) in the forced swim test in mice.

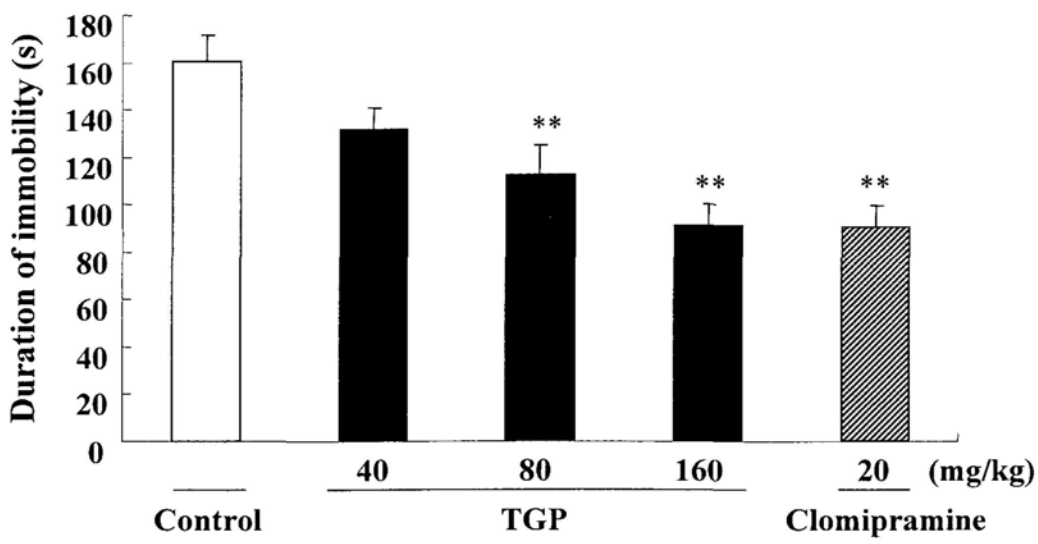


Figure 2.2 Effect of TGP on the duration of immobility in the forced swim test in mice.
Values given are the mean ± SEM (n = 10).
** p <0.01 as compared with control.

2.3.2 Effect of TGP on the duration of immobility in the forced swim test in rats

Figure 2.3 shows the effect of TGP treatment on the duration of immobility in the forced swim test in rats. Treating mice with TGP at the doses of 80 and 160 mg/kg for seven days significantly reduced the duration of immobility (41 % and 51%) in the forced swim test in mice, as compared to the control group. The classical antidepressant clomipramine, also caused a significant reduction in the immobility time (47%) in the forced swim test in mice.

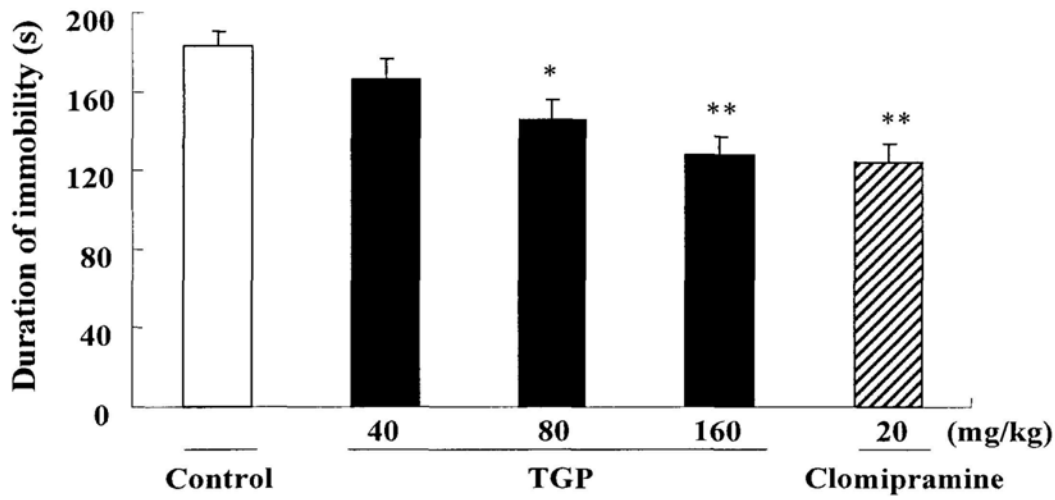


Figure 2.3 Effect of TGP on the duration of immobility in the forced swim test in rats.
Values given are the mean ± SEM (n = 10).
* p <0.05, ** p <0.01 as compared with control.

2.3.3 Effect of TGP on the duration of immobility in the tail suspension test in mice

Figure 2.4 shows the effect of TGP treatment on the duration of immobility in the tail suspension test in mice. Treating mice with TGP at the doses of 80 and 160 mg/kg for seven days significantly reduced the duration of immobility (41 % and 51%) in the tail suspension test in mice, as compared to the control group. The classical antidepressant clomipramine, also caused a significant reduction in the immobility time (47%) in the tail suspension test in mice.

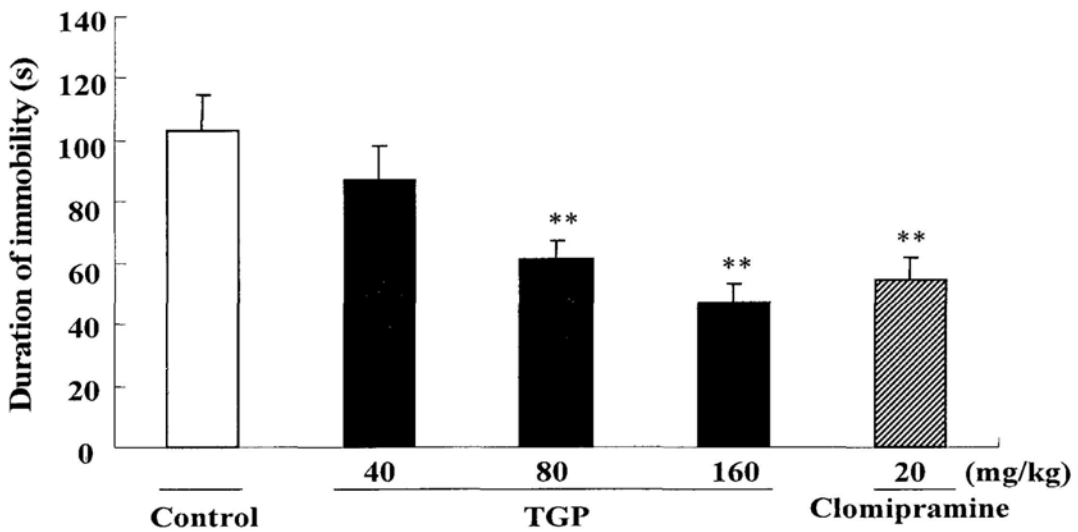


Figure 2.4 Effect of TGP on the duration of immobility in the tail suspension test in mice. Values given are the mean ± SEM (n = 10).
** p <0.01 as compared with control.

2.3.4 Effect of TGP on locomotor activity in the open-field test in mice

Table 2.1 shows the effect of TGP treatment on locomotor activity in mice. The locomotor activity was assessed by the number of crossings and rearings in the open-field test. TGP treatment at doses of 80 and 160 mg/kg caused a slight reduction in the number of crossings (23% and 29%) and rearings (23% and 25%) in the open-field test , as compared to the control group.

Table 2.1 Effect of TGP on the number of crossings and rearings in the open-field test in mice

Treatment	Number of crossings	Number of rearings
Control	99.7 ± 5.6	31.8 ± 2.0
TGP (40 mg/kg)	88.1 ± 5.9	26.6 ± 2.0
TGP (80 mg/kg)	77.1 ± 6.5 *	24.6 ± 1.6 *
TGP (160 mg/kg)	71.2 ± 6.3 **	23.8 ± 1.8 *
Clomipramine (20 mg/kg)	84.2 ± 6.1	26.2 ± 1.9

Values given are the mean ± SEM (n = 10).

* p <0.05, ** p <0.01 as compared with control.

2.3.5 Effect of TGP on locomotor activity in the open-field test in rats

Table 2.2 shows the effect of TGP treatment on locomotor activity in rats. TGP or clomipramine treatment produced no significant difference in the number of crossings (A) and rearings (B) in the open-field test in rats.

Table 2.2 Effect of TGP on the number of crossings and rearings in the open-field test in rats

Treatment	Number of crossings	Number of rearings
Control	49.7 ± 2.7	11.5 ± 1.1
TGP (40 mg/kg)	47.9 ± 3.3	10.5 ± 1.3
TGP (80 mg/kg)	45.8 ± 2.7	9.9 ± 1.0
TGP (160 mg/kg)	43.7 ± 3.0	9.5 ± 0.9
Clomipramine (20 mg/kg)	45.2 ± 2.9	10.1 ± 1.0

Values given are the mean ± SEM (n = 10).

2.4. Discussion

In this study, the antidepressive effects of TGP were evaluated by using forced swim test and tail suspension test. The forced swim test and tail suspension test are well established screening paradigms for antidepressants. In these two tests, animals are under stress from which they cannot escape. After an initial period of struggling, they would become immobile, resembling a state of despair and mental depression (Porsolt et al., 1977; Steru et al., 1985). Multiple classes of antidepressant drugs and several nonpharmacological antidepressant therapies, including transcranial magnetic stimulation, rapid eyemovement sleep deprivation and electroconvulsive shock, are known to reduce immobility time in the forced swim and tail suspension tests (Porsolt et al., 1977; Steru et al., 1985). Consistent with these, the present results showed that after being treated with TGP at 80 and 160 mg/kg for seven days, the mice showed a significant reduction of immobility time in both forced swim and tail suspension tests. Treated with TGP at 80 and 160 mg/kg for seven days also significantly reduced the duration of immobility in the forced swim test in rats.

In these behavioral tests, false-positive results can be obtained for agents that stimulate locomotor activity (Bourin et al., 2001). Therefore, the effect of TGP on locomotion was evaluated by the open-field test in mice and rats. The results showed that TGP or chlorimipramine treatment did not increase the number of crossings and rearings in the open-field test in both mice and rats. The finding suggested that the reduction of immobility time elicited by TGP treatment in the forced swim and tail suspension tests was unlikely due to a psychomotor-stimulant effect, but rather an antidepressant-like effect of TGP.

Chapter 3

Antidepressant-like effects of total glycosides of peony in chronic unpredictable mild stress-induced depression model in rats

3.1 Introduction

It is believed that chronic stress is a crucial factor involved in the onset and relapse of depression (Bidzinska, 1984; Sheline, 2000; Lee et al., 2002). In this regard, an animal model of chronic unpredictable mild stress (CUMS)-induced depression has been developed to simulate the pathogenesis of depression in humans. CUMS was found to induce long-term behavioral disturbances and neurochemical changes resembling the symptoms of clinical depression and has been generally thought to be the most promising and valuable depressive model in animals (Willner, 1997; Luo et al., 2008). The model has been developed by Willner et al. (1987) and employed in a number of antidepression studies (Papp et al., 2002; Li et al., 2007; Zhou et al., 2007a; Luo et al., 2008; Tõnissaar et al., 2008; Zhao et al., 2008).

Previous studies (Chapter 2) showed that the intragastric administration of total glycosides of peony (TGP) caused a significant reduction of immobility time in both forced swim and tail suspension tests in rodents. To further confirm the antidepressant-like effects of TGP, the present study aims to evaluate the antidepressant-like effects of TGP in CUMS-induced depression model in rats by using behavioral tests such as sucrose preference, open-field and forced swim tests.

3.2 Materials and methods

3.2.1 Drugs and chemical reagents

TGP was supplied by Ningbo Liwah Pharmaceutical Co., Ltd. (Zhejiang, China). Fluoxetine, a selective serotonin reuptake inhibitor, was purchased from Sigma-Aldrich (St. Louis, MO) and used as positive control for antidepressant action.

3.2.2 Animals

Male Sprague-Dawley rats weighing 200-220 g were obtained from the Laboratory Animal Services Center, The Chinese University of Hong Kong, Hong Kong. Animals were maintained on a 12-hour light/dark cycle (lights on at 6:00 AM, lights off at 6:00 PM) under controlled temperature (22 ± 2 °C) and humidity (50 ± 10%), and were given standard diet and water *ad libitum*. They were allowed to acclimatize for 7 days before use. The experiments on animals have been approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

3.2.3 CUMS procedure

Rats were randomly assigned into six groups of ten individuals: control (physiological saline), CUMS plus vehicle (physiological saline), CUMS plus TGP (80 mg/kg), CUMS plus TGP (160 mg/kg), and CUMS plus fluoxetine (20 mg/kg), and both TGP and fluoxetine were given intragastrically 30 min before each stressor once daily for 5 weeks. The CUMS procedure was performed as described by Zhao et al. (2008), with a slight modification. Briefly, CUMS consisted of a variety of unpredictable stressors, namely,

48-h food deprivation, 24-h water deprivation, 5-min cold swimming (at 6 °C), 1-min tail pinch (1 cm from the end of the tail), physically restraint for 2 h, exposure to a foreign object (e.g., a piece of plastic) for 24 h and overnight illumination. One of these stressors was given every day between 9:30 am to 12: 00 am for 5 weeks. The order of stressors used in present study was showed in Table 3.1. Control (unstressed) animals were undisturbed except for necessary procedures such as routine cage cleaning.

Table 3.1 Schedule of CUMS procedure

Day	Stressor	Day	Stressor
D1	5-min cold swimming (at 6 °C)	**D18**	overnight illumination
D2	1-min tail pinch	**D19**	Exposure to a foreign object for 24 h
D3	physically restraint for 2 h	**D20-21**	48-h food deprivation
D4	overnight illumination	**D22**	5-min cold swimming (at 6 °C)
D5	24-h water deprivation	**D23**	overnight illumination
D6	Exposure to a foreign object for 24 h	**D24**	1-min tail pinch
D7-D8	48-h food deprivation	**D25**	physically restraint for 2 h
D9	Exposure to a foreign object for 24 h	**D26**	24-h water deprivation
D10	overnight illumination	**D27**	overnight illumination
D11	5-min cold swimming (at 6 °C)	**D28**	5-min cold swimming (at 6 °C)
D12	physically restraint for 2 h	**D29**	physically restraint for 2 h
D13	5-min cold swimming (at 6 °C)	**D30-31**	48-h food deprivation
D14	1-min tail pinch	**D32**	Exposure to a foreign object for 24 h
D15	24-h water deprivation	**D33**	5-min cold swimming (at 6 °C)
D16	physically restraint for 2 h	**D34**	physically restraint for 2 h
D17	5-min cold swimming (at 6 °C)	**D35**	overnight illumination

3.2.4 Sucrose preference test

Sucrose preference test was carried out before stress and at the end of 5-week CUMS exposure. The test was performed as described previously (Luo et al., 2008) with minor modifications. Briefly, 72 h before the test, rats were trained to adapt 1% sucrose solution (w/v): two bottles of 1% sucrose solution were placed in each cage, and 24 h later 1% sucrose in one bottle was replaced with tap water for 24 h. After the adaptation, rats were deprived of water and food for 24 h. Sucrose preference test was conducted at 9:00 a.m. in which rats were housed in individual cages and were free to access to two bottles containing 100 mL of sucrose solution (1% w/v) and 100 ml of water, respectively. After 3 h, the volumes of consumed sucrose solution and water were recorded and the sucrose preference was calculated by the following formula:

$$\text{Sucrose preference} = \frac{sucrose\ consumption}{water\ consumption + sucrose\ consumption} \times 100\%.$$

3.2.5 Open-field test

Open-field test was carried out before stress and at the end of 5-week CUMS exposure. The test was performed as described previously (Zhao et al., 2008) with minor

modifications. The open-field apparatus consisted of a square wooden arena (100 cm ×100 cm × 40 cm) with black surface covering the inside walls. The floor of the wooden arena was divided equally into 25 squares marked by black lines. In the test, the rat was placed individually into the center of the arena and allowed to explore freely. The number of crossings (squares crossed with all paws) and rearings (rising of the front paws) were recorded during a test period of 3 min. This apparatus was cleaned with a detergent and dried after occupancy by each rat.

3.2.6 Forced swim test

Forced swim test was carried out before stress and at the end of 5-week CUMS exposure on 2 successive days. The test was performed according to a modification of the traditional method described by Porsolt et al. (1977), as suggested by Li et al. (2003) for rats. Briefly, rats were forced to swim in a vertical plastic cylinder (diameter 21 cm, height 50 cm) containing 25 cm of water maintained at 25 ± 1 °C. On the 1st day of experiment, rats were forced to swim for 15 min. They were then dried and returned to their home cages. On the following day, rats were re-exposed to the forced swimming for 5 min. The total duration of immobility (seconds) was quantified during a test period

of 5 min. Rats were considered immobile when they made no attempts to escape except the movements necessary to keep their heads above the water.

3.2.7 Statistical analysis

Data were expressed as mean ± SEM. The GraphPad Prism software (version 4.0) was used to perform the statistics (GraphPad Software, Inc., San Diego, CA). One-way analysis of variance (ANOVA) followed by Dunnett's test was used to test the differences between the groups. Difference was considered statistically significant when the value $p < 0.05$.

3.3 Results

3.3.1 Effect of TGP on the percentage of sucrose consumption in CUMS-treated rats

Figure 3.1 shows the effect of TGP treatment on the percentage of sucrose consumption in CUMS-treated rats. Before CUMS treatment was given, there was no significant change in the percentage of sucrose consumption among all rats. A 5-week CUMS exposure significantly reduced the percentage of sucrose consumption in the animals (43%), as compared to the control (i.e., non-CUMS-treated rats). While long-term treatment with TGP at daily doses of 80 or 160 mg/kg significantly increased the percentage of sucrose consumption in CUMS-treated rats (25% and 34%, respectively), as compared to the vehicle-treated and CUMS-treated rats. Treatment with fluoxethine (20 mg/kg) also significantly increased the percentage of sucrose consumption in CUMS-treated rats (44%).

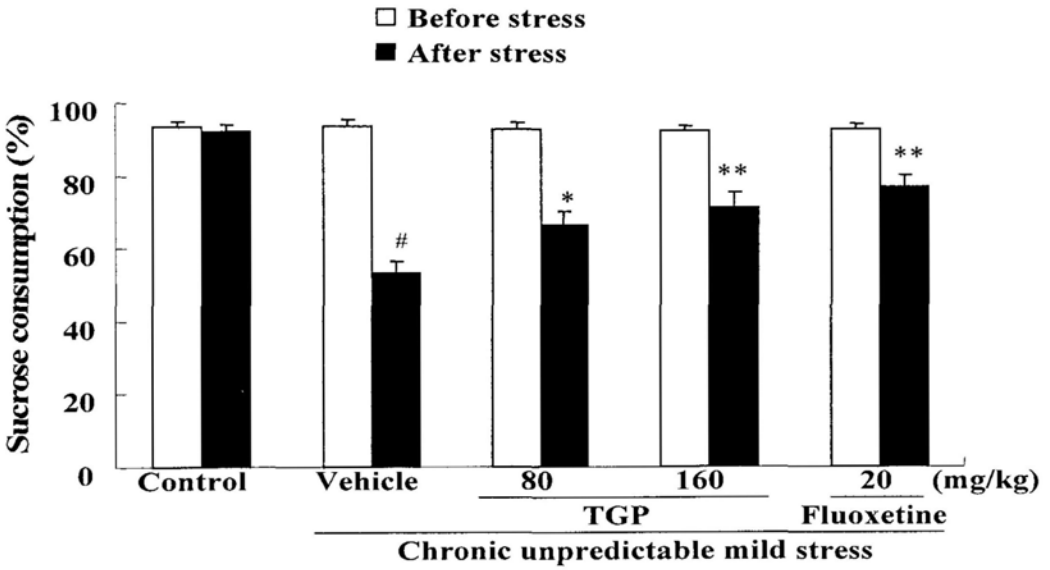


Figure 3.1 Effect of TGP on the percentage of sucrose consumption in CUMS-treated rats. Values given are the mean ± SEMs (n = 10).

$^{\#}p < 0.01$ as compared with the control; $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with the vehicle-treated CUMS group.

3.3.2 Effect of TGP on locomotor activity in the open-field test in CUMS-treated rats

Figure 3.2 shows the effect of TGP treatment on locomotor activity of the CUMS-treated rats in the open-field test. The locomotor activity was assessed by the number of crossings and rearings in the open-field test. Before CUMS treatment was given, there was no significant change in the number of crossings and rearings among all rats. A 5-week CUMS exposure significantly reduced the number of crossings (A) and rearing (B) in rats (70% and 62%, respectively), as compared to the control. Long-term treatment with TGP at daily doses of 80 or 160 mg/kg significantly increased the number of crossings in CUMS-treated rats (53% and 73%, respectively), as compared to the vehicle-treated and CUMS-treated rats. Long-term treatment with TGP at a daily dose of 160 mg/kg also significantly increased the number of rearings in CUMS-treated rats (56%), as compared to the vehicle-treated and CUMS-treated rats. Treatment with fluoxetine (20 mg/kg) as well significantly increased the number of crossings and rearings in CUMS-treated rats (87% and 60%, respectively).

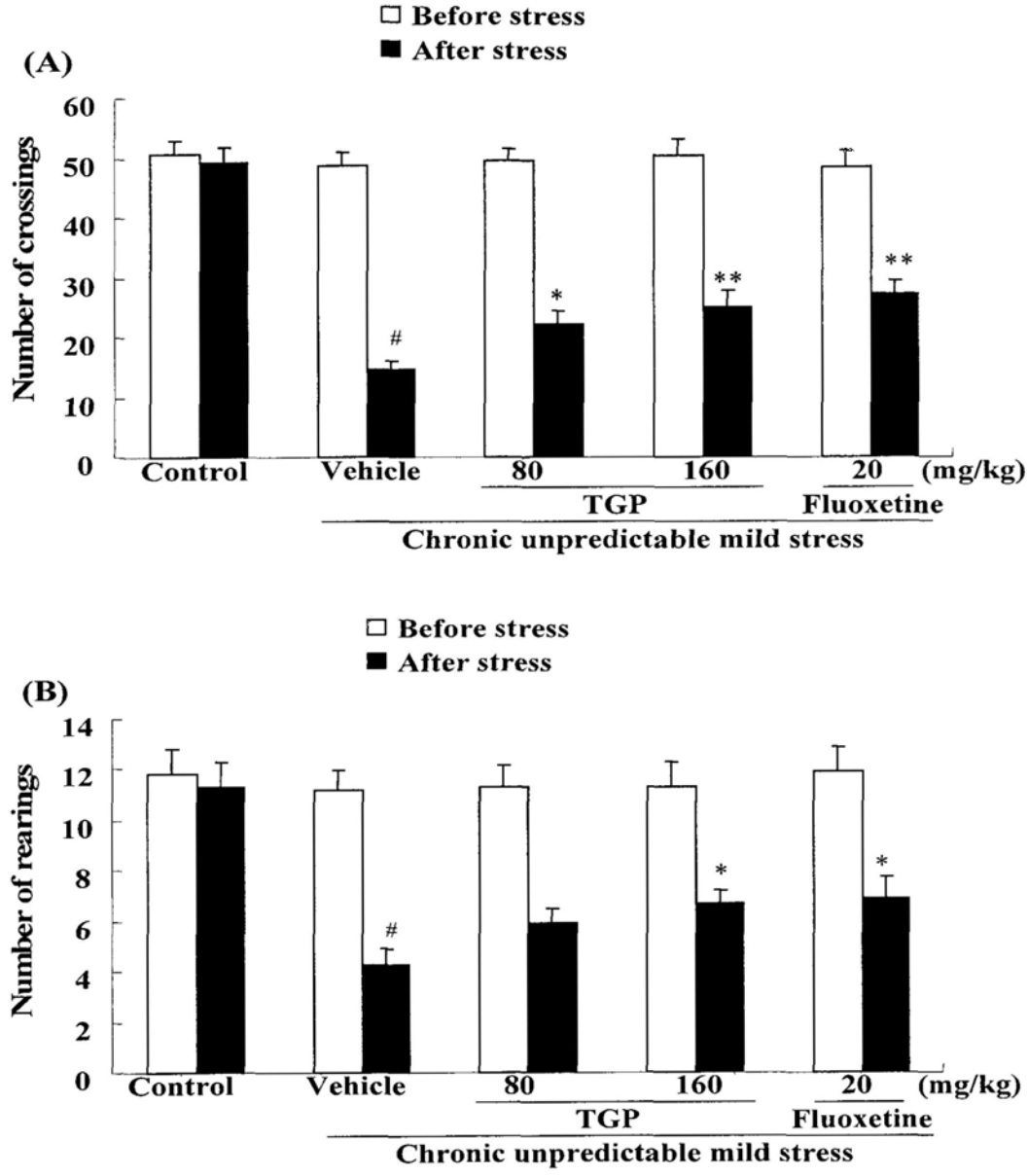


Figure 3.2 Effect of TGP on the number of crossings (A) and rearings (B) in the open-field test in CUMS-treated rats.

Values given are the mean ± SEMs (n = 10).

$^{\#}p < 0.01$ as compared with the control; $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with the vehicle-treated CUMS group.

3.3.3 Effect of TGP on the immobility time in the forced swim test in CUMS-treated rats

Figure 3.3 shows the effect of TGP treatment on the immobility time of rats in the forced swim test. Before CUMS treatment was given, there was no significant change in the immobility time in the forced swim test among all rats. The exposure to CUMS caused a significant increase in the immobility time of rats (37%), as compared to the control. TGP treatment at doses of 80 or 160 mg/kg significantly decreased the immobility time of rats exposed to CUMS in a dose-dependent manner (16% and 22%, respectively), as compared to the vehicle-treated and CUMS-treated rats. Fluoxetine treatment at a dose of 20 mg/kg also significantly decreased the immobility time of CUMS-treated rats (25%).

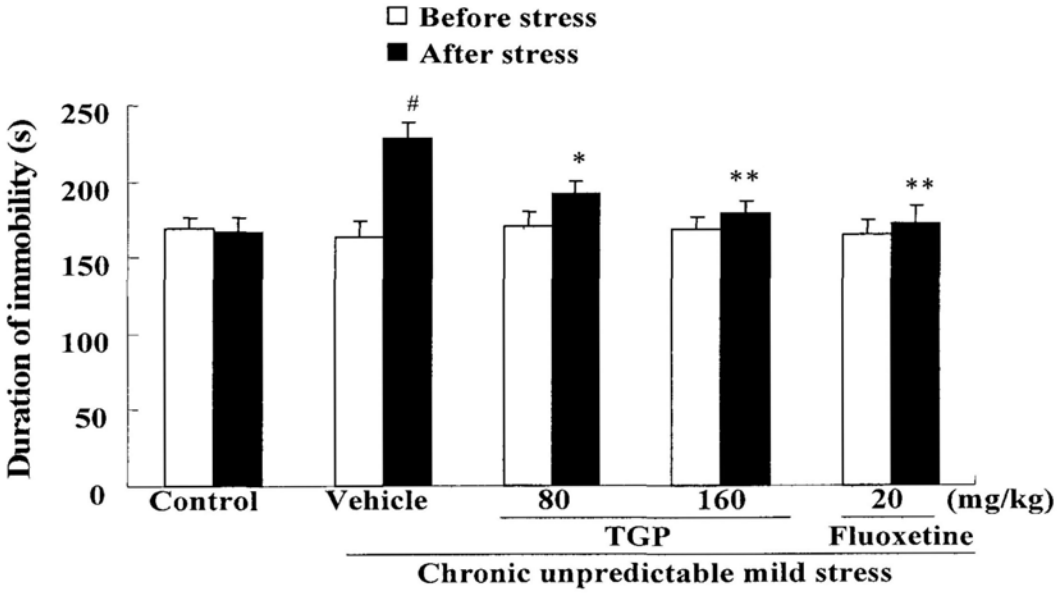


Figure 3.3 Effect of TGP on the immobility time in the forced swim test in CUMS-treated rats.

Values given are the mean ± SEMs (n = 10).

$^{\#}p < 0.01$ as compared with the control; $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with the vehicle-treated CUMS group.

3.4 Discussion

The experimental model of CUMS-induced depression is relevant to clinical depression, and many behavioral and biochemical changes observable in animals are paralleled by alterations in patients with depression (Willner, 1997 and 2005). It has been shown that the CUMS-induced depression model can be used to evaluate the efficacy of drug antidepressant candidates through behavioral tests such as sucrose preference, open-field and forced swim tests (Papp et al., 2002; Li et al., 2007; Zhou et al., 2007a; Tõnissaar et al., 2008; Zhao et al., 2008).

Sucrose preference test is an indicator of anhedonia-like behavioral change (Willner, 1997 and 2005). Anhedonia, a core symptom of human major depression, was modeled by inducing a decrease in responsiveness to rewards reflected by a reduced consumption and/or preference of sweetened solutions (Willner, 1997 and 2005). The results of present study showed that rat subjected to CUMS procedure consumed less sucrose solution when compared to non-stressed rats. Long-term treatment of TGP significantly suppressed this behavioral change which suggested the antidepressant-like action of TGP.

In the open-field test, normal animals usually show increased locomotor activity in a novel open field, which is driven by the instinct of exploration in a novel environment (Luo et al., 2008). However, after chronic stress, animal displayed a decreased locomotor activity in a novel open field (Katz et al., 1981; D'Aquila et al., 2000), indicative of a behavioral change that may reflect certain aspects of "refractory loss of interest" (Katz et al., 1981; Luo et al., 2008). In our present study, we are able to demonstrate that CUMS, for a period of 5 weeks, significantly reduced the number of crossings and rearing in the open-field test. The CUMS-induced decrease in locomotor activity was ameliorated by long-term TGP treatment during the course of CUMS.

Since 1977 when the original report by Porsolt and colleagues was published, forced swim test has been widely used for assessing the effectiveness of candidate antidepressants. Chronic stress has been shown to dramatically increase the immobility time of mice in forced swim test (Zhou et al., 2007a; Tõnissaar et al., 2008), a manifestation of "behavioral despair" (Cryan et al., 2005). Consistently, CUMS, as observed in the present study, resulted in an increased immobility time in forced swim test in mice. Long-term TGP treatment also counteracted the CUMS-induced increase in immobility time in rats.

Taken together, results obtained from behavioral studies indicated that long-term TGP treatment produced an antidepressant-like action in CUMS-induced depression model in rats.

Chapter 4

Antidepressive and neuroprotective mechanisms of total glycosides of peony in rats treated with chronic unpredictable mild stress

4.1 Introduction

Previous studies (Chapter 2) showed that the intragastric administration of total glycosides of peony (TGP) caused a significant reduction of immobility time in both forced swim and tail suspension tests in rodents. Long-term treatment with TGP also produced antidepressant-like effects in the chronic unpredictable mild stress (CUMS)-induced depression model in rats (Chapter 3). However, the mechanism (s) underlying the antidepressant-like action of TGP are still unknown.

The hippocampus has been evidenced as a key structure of the brain in pathophysiology of depression/stress disorders. Several recent studies showed that major depression was closely associated with reduced hippocampal volumes, and there was a positive correlation between the hippocampus atrophy and the time course of depression (Li et al., 2003b; MacQueen et al., 2003; Saylam et al., 2006). Postmortem analyses indicated a reduction

in the neuronal cells in the hippocampus of patients with major depression (Stockmeier et al., 2004). On the other hand, it has been reported that antidepressants possessed neuroprotective properties, and they were able to ameliorate impairments in cellular plasticity and resilience in the hippocampus (Bachmann et al., 2005). These data suggest that neuronal atrophy and destruction in the hippocampus plays a causal role in the development and progress of depression and protect against hippocampal neuronal damage may be an important therapeutic intervention of depression (Manji and Duman, 2001; Fuchs et al., 2004).

The CUMS-induced depression model is generally thought to be the most promising and valuable rodent model to study depression in animals, mimicking several human depressive symptoms. Moreover, as proposed by Willner (Willner, 1997 and 2005), CUMS appears to be more suitable for studying the neurobiological basis of depression and the mechanisms of action of antidepressant drugs compared to the acute stress models. The present study aims to examine the neuroprotective effects of TGP on the hippocampus of CUMS-treated rats. In order to investigate the mechanism(s) underlying the neuroprotective effects of TGP, serum corticosterone level, and glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) mRNA levels, intracellular reactive oxygen

species (ROS) level, reduced glutathione (GSH) level, malondialdehyde (MDA) level, superoxide dismutase (SOD) activity, catalase (CAT) activity, neurotrophins such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin-3 (NT-3) protein and mRNA levels in the hippocampus were measured in CUMS-treated rats, with or without TGP treatment.

4.2 Materials and methods

4.2.1 Drugs and chemical reagents

TGP was supplied by Ningbo Liwah Pharmaceutical Co., Ltd. (Zhejiang, China). 2',7'-Dichlorofluorescin diacetate (DCFH-DA) was obtained from Invitrogen (Carlsbad, CA, USA). 5,5'-Dithiobis-2-nitrobenzoic acid, toluidine blue and trichloroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents and solvents were of analytical grade.

4.2.2 Animals

Male Sprague-Dawley rats weighing 200-220 g were obtained from the Laboratory Animal Services Center, The Chinese University of Hong Kong, Hong Kong. Animals were maintained on a 12-hour light/dark cycle (lights on at 6:00 AM, lights off at 6:00 PM) under controlled temperature (22 ± 2 °C) and humidity (50 ± 10%), and were given standard diet and water *ad libitum*. They were allowed to acclimatize for 7 days before use. The experiments on animals have been approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong and conformed to the guidelines of the "Principles of Laboratory Animal Care" (NIH publication No.80-23, revised 1996). Effort was made to minimize the number and suffering of the animals.

4.2.3 CUMS procedure

Rats were randomly assigned into four groups of twelve individuals: control, CUMS plus vehicle (physiological saline), CUMS plus TGP (80 mg/kg) and CUMS plus TGP (160 mg/kg). TGP were dissolved in physiological saline and given intragastrically 30 min before each stressor once every day for 5 weeks. The CUMS procedure was performed

as described by Zhao et al. (2008), with a slight modification. Briefly, CUMS consisted of a variety of unpredictable stressors, namely, 48-h food deprivation, 24-h water deprivation, 5-min cold swimming (at 6 °C), 1-min tail pinch (1 cm from the end of the tail), physically restraint for 2 h, exposure to a foreign object (e.g., a piece of plastic) for 24 h and overnight illumination. One of these stressors was given every day between 9:30 am to 12: 00 am for 5 weeks. The order of stressors used was the same as showed in Chapter 3 (Table 3.1). Control (unstressed) animals were undisturbed except for necessary procedures such as routine cage cleaning.

4.2.4 Histological analysis

Twenty-four hours after the last stressor, rats (n=4/group) were sacrificed by decapitation and thier brain samples were immediately dissected out, fixed in 10% buffered formalin for 1 week, dehydrated with alcohols, and embedded in paraffin. Tissue sections (10 μm thick) containing hippocampus were prepared using a Leica radial microtomes and mounted on gelatin-coated glass microscope slides. After drying at room temperature, the sections were de-paraffinized in xylen, rehydrated, and stained with toluidine blue. To assess hippocampal injury, Nissl-positive neuronal cell numbers were manually and

rigidly counted in the hippocampal CA3 region under a light microscope at high magnification (×400) as previously described (Liu et al., 2006; Xu et al., 2009). The total cell counts were averaged from four different fields per animal. Data was expressed as surviving cell number per millimeter (mm) in the CA3 region.

4.2.5 Blood sampling and tissue collection

Twenty-four hours after the last stressor, rats (n= 8/group) were sacrificed by decapitation and the blood samples were collected in tubes. Serum was separated by centrifugation at 4000 g for 10 min and stored at -80 °C until assay. Whole brains were rapidly removed from rats and chilled in an ice-cold saline solution. Hippocampus samples were dissected on a cold plate according to the method of Angelucci et al. (2003) and then stored at -80 °C until assay.

4.2.6 Measurement of corticosterone level in serum

Serum corticosterone level was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Assay Designs, Inc., Michigan) according to the

manufacturer's protocol. Briefly, samples, serial dilutions of corticosterone standard (0-20000 pg/mL), corticosterone EIA conjugate and corticosterone EIA antibody were applied into a 96-well plate precoated with donkey anti-Sheep IgG and incubated on a shaker for 2 h at room temperature. Then pNpp substrate solution was added and incubated at room temperature for 1 hour after three washes. The reaction was stopped with trisodium phosphate and absorbance was recorded at 405 nm immediately. Corticosterone level was determined from the the standard calibration curve constructed under same conditions. The detection limit of the assay is ~ 27 pg/mL. Data were expressed as ng/mL and all assays were performed in duplicate.

4.2.7 Biochemical analysis

4.2.7.1 Preparation of tissue homogenates

Hippocampus samples were homogenized in chilled phosphate buffer (pH 7.4). The homogenates were centrifuged at 800 × g for 5 min at 4 °C to separate the nuclear debris. The supernatant thus obtained was centrifuged at 10,000 ×g for 20 min at 4 °C to get the post mitochondrial supernatant (PMS), which was used to assay the levels of intracellular

ROS, MDA and GSH, and the activites of SOD and CAT.

4.2.7.2 Measurement of intracellular ROS level

The Intracellular ROS level was measured as previously described (Dohare et al., 2008). Briefly, an aliquot (100 μL) of PMS was incubated with DCFH-DA at a final concentration of 10 μM for 30 min at 37 °C in darkness. The fluorescence intensity was then measured in a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The ROS level was normalized to the protein concentration for each sample and expressed as a percentage of non-stressed control.

4.2.7.3 Measurement of MDA content

MDA content was measured by the method of Ohkawa et al. (1979), using thiobarbituric acid. Briefly, an aliquot (100 μL) of PMS was mixed with 1500 μL of acetic acid (20% [v/v], pH 3.5), 1500 μL of thiobarbituric acid (0.8 %, w/v) and 200 μL of sodium dodecyl sulfate (8%, w/v). Each reaction mixture was heated for 60 min at 95 °C and cooled to room temperature. Next, 5000 μL of n–butanol was added. After mixing and

centrifugation at 3000 *g* for 10 min, the organic layer was collected and the absorbance of the solution was measured at 532 nm. The MDA content was normalized to the protein concentration for each sample and expressed as nmol/mg protein.

4.2.7.4 Measurement of GSH content

GSH content was measured according to the method of Gulati et al. (2007). Briefly, an aliquot (100 μL) of PMS was mixed with 200 μL of trichloroacetic acid (25%, v/v) and 200 μL of saline. The mixture was centrifuged (3000 *g*) for 10 min at 4 °C. An aliquot (200 μL) of supernatant was mixed with 1 mL of phosphate buffer (100 mM, pH 8.0) and 50 μL of 5,5'-Dithiobis-2-nitrobenzoic acid (3 mM). The solution was stood at room temperature for 5 minutes and its absorbance was measured at 412 nm. The GSH content was normalized to the protein concentration for each sample and expressed as nmol/mg protein.

4.2.7.5 Measurement of SOD activity

SOD activity was measured using a SOD activity assay kit (BioVision, Mountain View, CA) according to the manufacturer's protocol. Briefly, 20 μL of PMS was added with 200 μL of water soluble tetrazolium working solution and 20 μL of enzyme working solution into a 96-well plate. After incubating the plate at 37 °C for 20 min, the absorbance of each well was measured at 450 nm by using a microplate reader. The SOD activity was normalized to the protein concentration for each sample and expressed as nU/mg protein.

4.2.7.6 Measurement of CAT activity

CAT activity was measured according to the method of Shen et al. (2009). Briefly, 50 μL of PMS was mixed with 1950 μL of phosphate buffer (0.05 M, pH 7.0) and 1000 μL of hydrogen peroxide (0.019 M). The activity of CAT was calculated by the decrease of absorbency at 240 nm over 2 min. The CAT activity was normalized to the protein concentration for each sample and expressed as U/g protein.

4.2.7.7 Measurement of protein concentration

The protein content was measured according to the method of Lowry (Lowry et al., 1951) using bovine serum albumin as standard.

4.2.8 Measurement of GR, MR, BDNF, NGF and NT-3 mRNA levels

GR, MR, BDNF, NGF and NT-3 mRNA levels were measured using TaqMan real-time reverse transcription-PCR analysis. Briefly, total RNA from hippocampus was isolated with TRIzol Reagent (Gibco, Grand Island, NY) according to the manufacturer's protocol. Concentrations of extracted RNA were calculated from the absorbance at 260 nm. The quality of RNA was assessed by absorbances at 260 nm and 280 nm, with the ratio of A_{260} to A_{280} ranging from 1.9 to 2.1 being acceptable. Total RNA (1.5 μg) was transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's protocol. Assays-on-Demand primers for GR (Rn00561369_m1), MR (Rn00565562_m1), BDNF (Rn02531967_s1), NGF (Rn01533872_m1), NT-3 (Rn00579280_m1) and β-actin (Rn00667869_m1) were purchased from Applied Biosystems, Inc. (Foster City, CA). Real-time quantitative PCR

analysis was performed with a TaqMan® Fast Universal PCR Master Mix Kit (Applied Biosystems, Inc., Foster City, CA) by using StepOnePlus™ Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA) with the following profile: 2 min hold at 50 °C, 10 min hold at 95 °C, followed by 60 cycles of 15 sec at 95 °C and 1 min at 60 °C. Sequence Detection Software 2.0 (Applied Biosystems, Inc., Foster City, CA) was used for data analysis. The relative expression of GR, MR, BDNF, NGF and NT-3 mRNA was normalized to the amount of β-actin in the same cDNA using the relative quantification method ($2^{-\Delta\Delta CT}$ method) as described by the manufacturer.

4.2.9 Measurement of BDNF, NGF and NT-3 protein levels

Hippocampus samples were weighed and homogenized in tenfold volume of lysis buffer. The homogenate was then centrifuged at 10,000 g for 30 min at 4 °C and supernatants were used for BDNF, NGF and NT-3 assays.

Protein levels of BDNF were measured using a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kit (Chemicon International, Temecula, CA) according to the manufacturer's instructions. Briefly, samples and serial dilutions of

BDNF standard (0-500 pg/mL) were applied into 96-well immunoplates precoated with rabbit anti-human BDNF antibody and incubated on a shaker overnight at 4 °C. After washing four times, biotinylated mouse anti-human BDNF antibody was added and incubated for 3 h at room temperature. Then streptavidin-HRP conjugate solution was added and incubated at room temperature for 1 hour after washing. TMB/E substrate was added and incubated at room temperature for 15 minutes. The reaction was stopped with 1 M hydrochloric acid and absorbance was recorded at 450 nm immediately. BDNF concentration was determined from the standard calibration curve. The detection limit of the assay is 7.8 pg/mL, and the cross-reactivity with other related neurotrophic factors (NGF, NT-3 and Neurotrophin-4) was not observed. Data were represented as ng/g wet weight.

Protein level of NGF was measured using a two-site ELISA kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 96-well immunoplate was coated with anti-NGF pAb (capture Ab). After overnight incubation at 4 °C, the plate was blocked with "Block and Sample" buffer for 1 h. Then samples and serial dilutions of NGF standard (0-250 pg/mL) were added after washing and then shaken for 6 h at room temperature. After three washes, anti-NGF mAb was added and incubated overnight at 4

°C. Then anti-rat IgG HRP-conjugate was added and incubated for 2.5 h at room temperature under orbital shaking. The substrate, "TMB One Solution" as added. The reaction was stopped with 1 M hydrochloric acid and absorbance was recorded at 450 nm within 30 minutes. NGF concentrations were determined from NGF standard calibration curve constructed under same conditions. The detection limit of the assay is ~ 7.8 pg/mL, and the cross-reactivity with other related neurotrophic factors (BDNF, NT-3 and Neurotrophin-4) was less than 3%. Data were represented as ng/g wet weight.

Protein levels of NT-3 were measured using a two-site ELISA kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 96-well immunoplates were coated with anti-NT-3 pAb (capture Ab). After overnight incubation at 4 °C, the plate was blocked with "Block and Sample" buffer for 1 h. Then samples and serial dilutions of NGF standard (0-300 pg/mL) were added after washing and then shaken for 6 h at room temperature. After three washes, the anti-NT-3 mAb was added and incubated overnight at 4 °C. Then anti-rat IgG HRP-conjugate was added and incubated for 2.5 h at room temperature under orbital shaking. The substrate, "TMB One Solution" as added. The reaction was stopped with 1 M hydrochloric acid and absorbance was recorded at 450 nm. NT-3 concentrations were determined from NT-3 standard calibration curve constructed

under same conditions. The detection limit of the assay is 9.4 pg/mL, and the cross-reactivity with other related neurotrophic factors (BDNF, NGF and Neurotrophin-4) was less than 3%. Data were represented as ng/g wet weight.

4.2.10 Statistical analysis

Data were expressed as mean ± SEM. The GraphPad Prism software (version 4.0) was used to perform the statistics (GraphPad Software, Inc., San Diego, CA). One-way analysis of variance (ANOVA) followed by Dunnett's test was used to test the differences between the groups. Difference was considered statistically significant when the value $p < 0.05$.

4.3 Results

4.3.1 Effect of TGP on pyramidal neuronal cell loss in the hippocampal CA3 region of CUMS-treated rats

Nissl staining is a histological method to detect the structural abnormalities of the hippocampus (Figure 4.1). The arrangement of pyramidal neurons in the hippocampal CA3 region of the control group was trim and dense (Figure 4.1A). In contrast, the arrangement of pyramidal neurons in the hippocampal CA3 region of the vehicle-treated and CUMS group was sparse (Figure 4.1B). TGP treatment at doses of 80 and 160 mg/kg could improve CUMS-induced abnormal-arranged pyramidal neurons in the hippocampal CA3 region of rats (Figure 4.1C and Figure 4.1D, respectively). Figure 4.2 shows the number of pyramidal neuronal cells in the hippocampal CA3 region. A 5-week CUMS exposure significantly decreased the number of pyramidal neuronal cells in the hippocampal CA3 region by 46% when compared with the control. TGP treatment dose-dependently increased the number of neuronal cells in the hippocampal CA3 region of CUMS-treated rats (35-49%), as compared to the vehicle-treated and CUMS-treated rats.

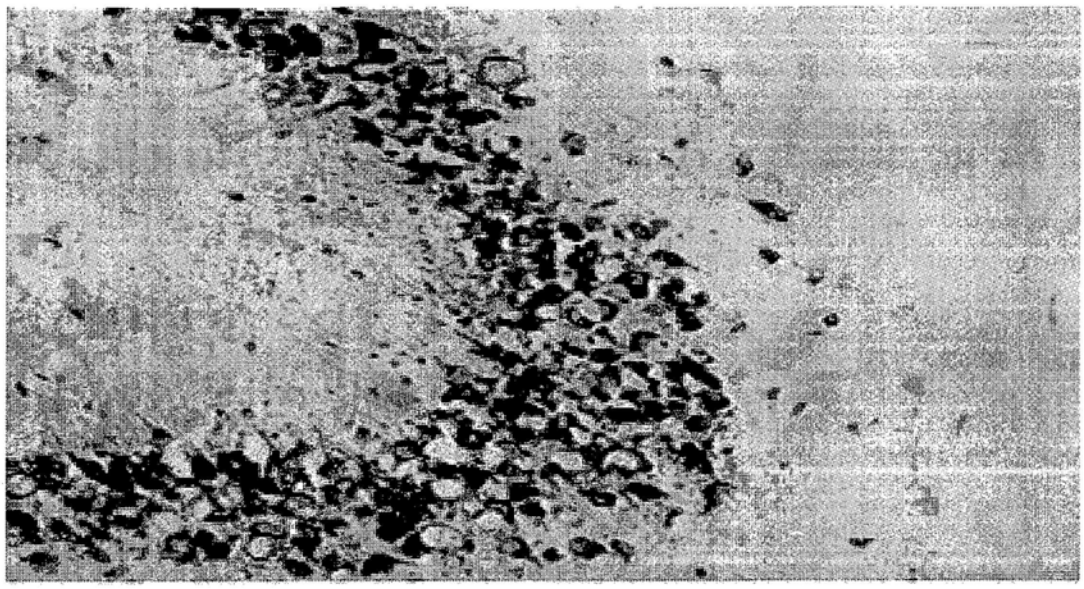


Figure 4.1A Pyramidal neurons in the hippocampal CA3 region of the control group (Nissl staining, magnification ×200).

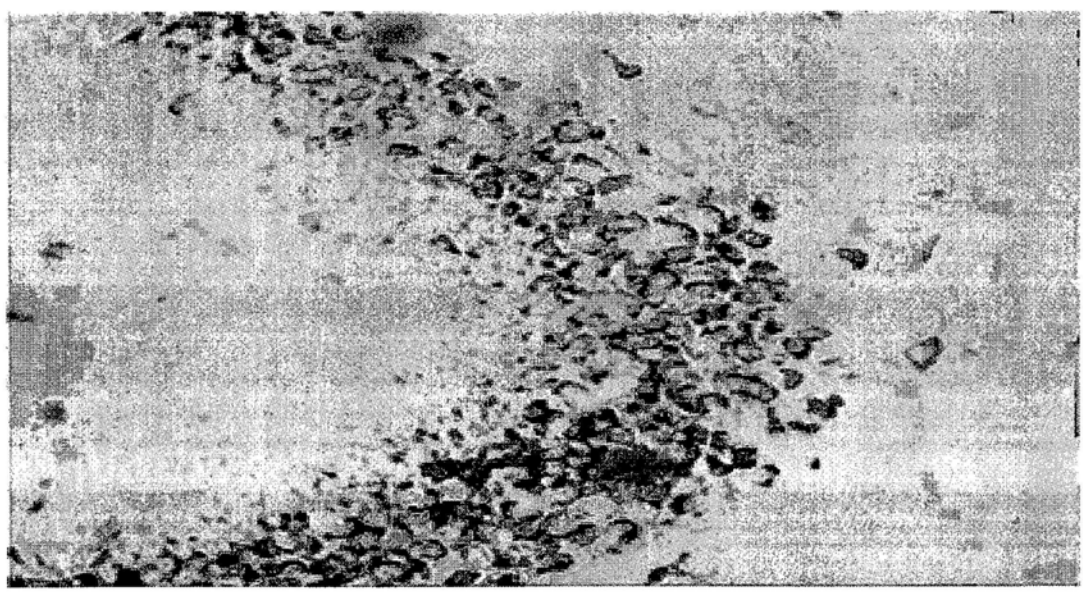


Figure 4.1B Pyramidal neurons in the hippocampal CA3 region of the vehicle plus CUMS group ((Nissl staining, magnification ×200).

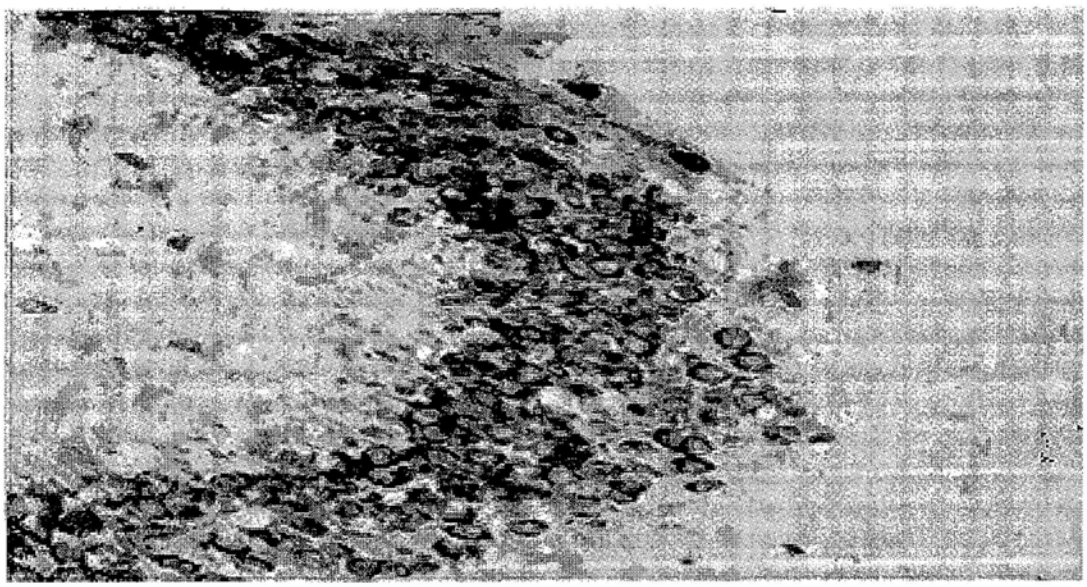


Figure 4.1C Pyramidal neurons in the hippocampal CA3 region of the TGP (80 mg/kg) plus CUMS group ((Nissl staining, magnification ×200).

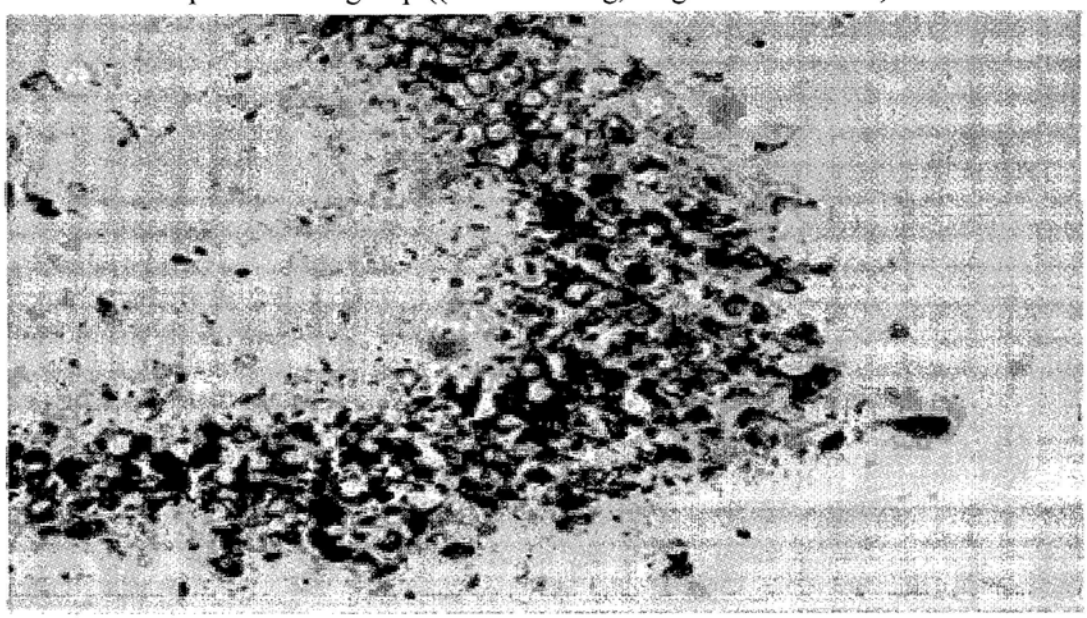


Figure 4.1D Pyramidal neurons in the hippocampal CA3 region of the TGP (160 mg/kg) plus CUMS group ((Nissl staining, magnification ×200).

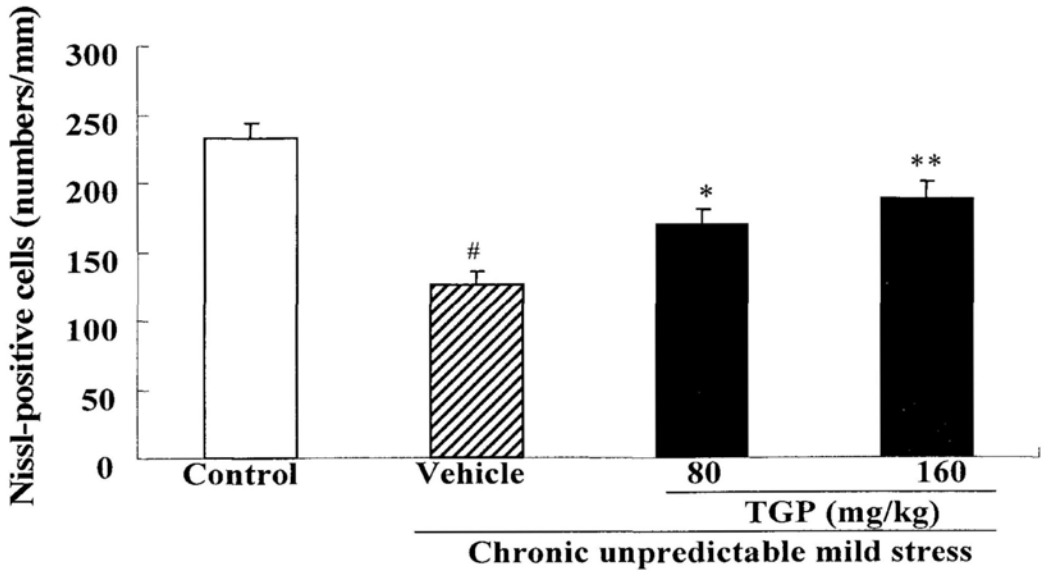


Figure 4.2 Quantitative analysis of the number of pyramidal neuronal cells in the hippocampal CA3 region.

Values given are the mean ± SEMs (n = 4).

$^{\#}p < 0.01$ as compared to the control; $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to the vehicle-treated CUMS group.

4.3.2 Effect of TGP on serum corticosterone level in CUMS-treated rats

Figure 4.3 shows the effect of TGP treatment on serum corticosterone level in CUMS-treated rats. A 5-week CUMS exposure caused a significant increase in serum corticosterone level in rats (223%), as compared to the control. TGP treatment dose-dependently decreased serum corticosterone level in CUMS-treated rats (15-24%), as compared to the vehicle-treated and CUMS-treated rats.

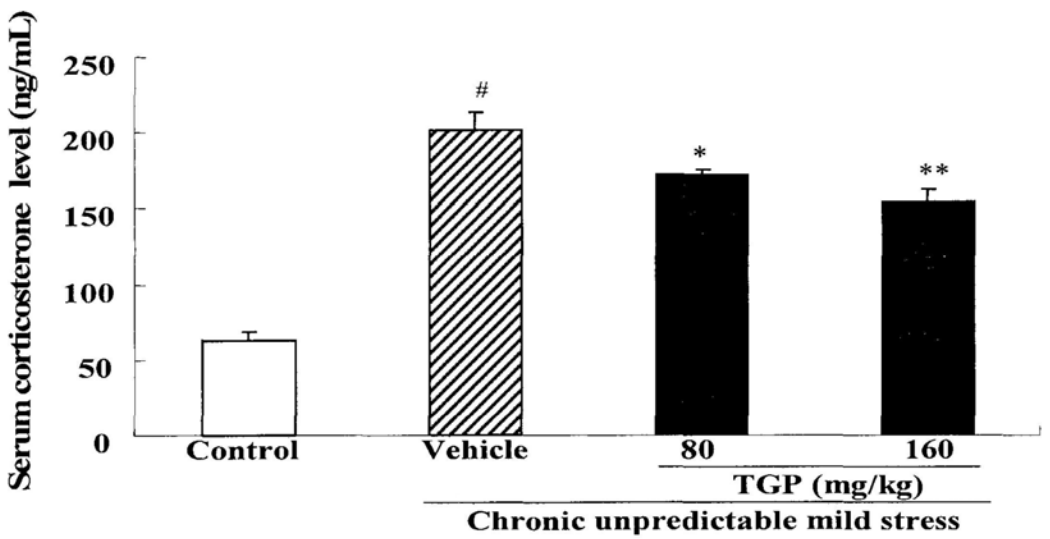


Figure 4.3 Effect of TGP on serum corticosterone level in CUMS-treated rats. Values given are the mean ± SEMs (n = 8).
$^{\#}p$ <0.01 as compared to the control; $^{*}p$ <0.05, $^{**}p$ <0.01 as compared to the vehicle-treated CUMS group.

4.3.3 Effect of TGP on GR and MR mRNA levels in the hippocampus of CUMS-treated rats

Effects of TGP treatment on GR and MR mRNA levels in the hippocampus of CUMS-treated and native rats are showed in Figure 4.4A and Figure 4.4B respectively. A 5-week CUMS exposure significantly decreased the hippocampal GR mRNA level in rats (54%), as compared to the control. TGP (80 and 160 mg/kg) treatment significantly increased GR mRNA level in the hippocampus of CUMS-treated rats (26% and 69%, respectively), as compared with he vehicle-treated and CUMS-treated rats. On the other hand, there was no significant change in the MR mRNA level in the hippocampus among all rats.

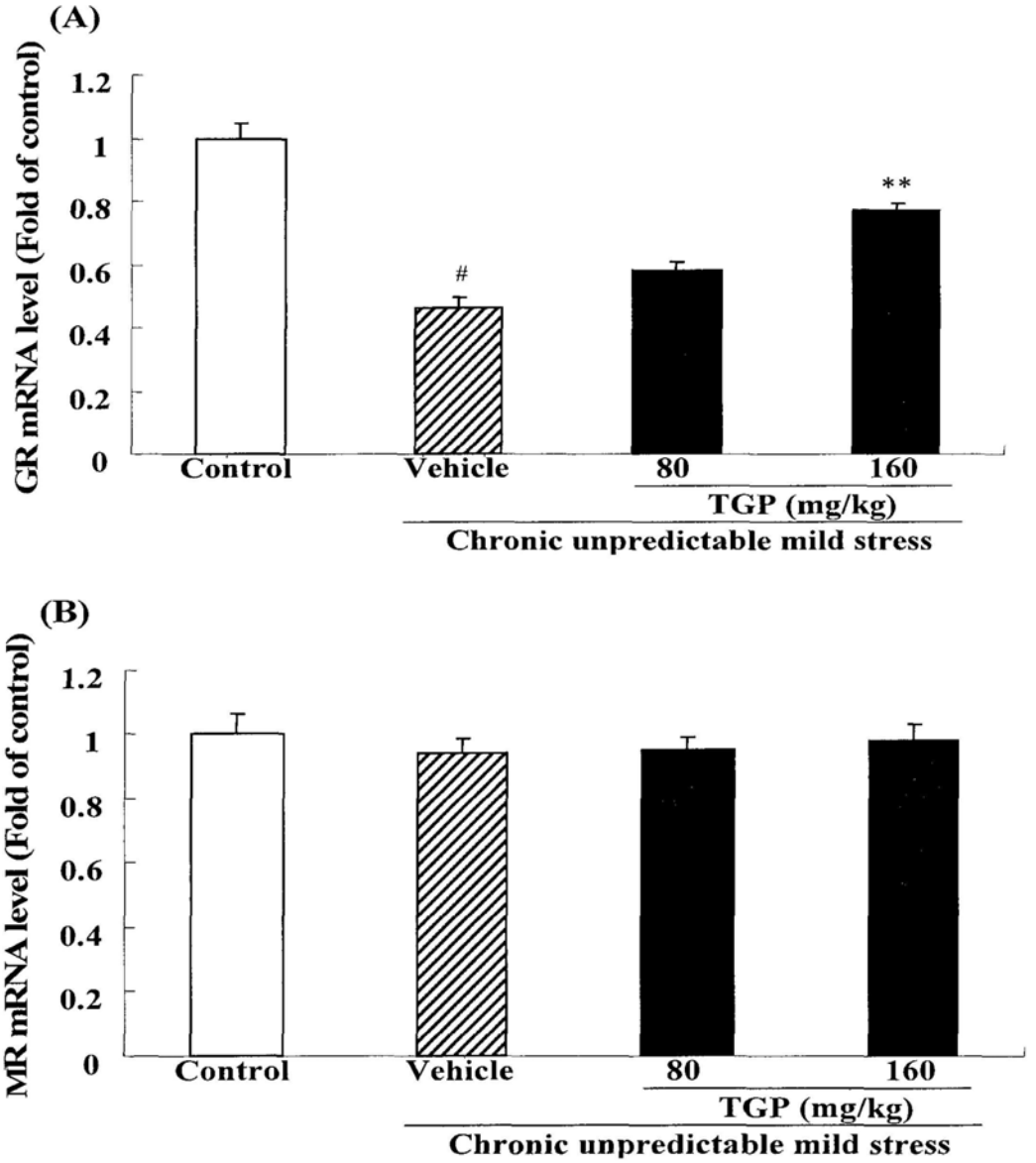


Figure 4.4 Effect of TGP on GR (A) and MR (B) mRNA levels in the hippocampus of CUMS-treated rats.

Values given are the mean ± SEMs (n = 6).

$^{\#}p < 0.01$ as compared to the control; $^{**}p < 0.01$ as compared with the vehicle-treated CUMS group.

4.3.4 Effect of TGP on intracellular ROS level in the hippocampus of CUMS-treated rats

As shown in Figure 4.5, 5-week CUMS exposure significantly increased intracellular ROS level in the hippocampus of rats (83%), as compared to the control. TGP (80 and 160 mg/kg) treatment significantly decreased intracellular ROS level in the hippocampus of CUMS-treated rats (21% and 29%, respectively), as compared with the vehicle-treated and CUMS-treated rats.

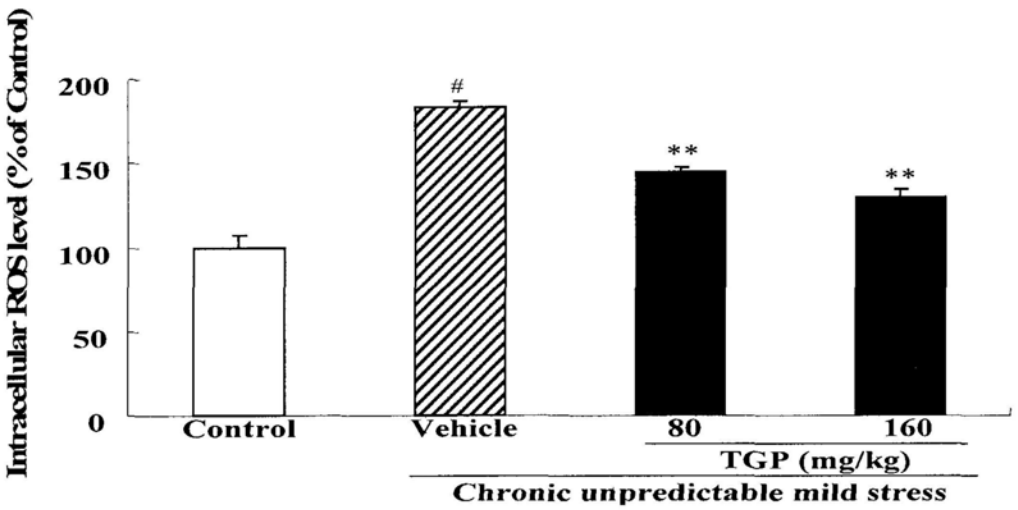


Figure 4.5 Effect of TGP on intracellular ROS level in the hippocampus of CUMS-treated rats.

Values given are the mean ± SEMs (n =8).

$^{\#}p$ <0.01 as compared to the control; $^{**}p$ <0.01 as compared with the vehicle-treated CUMS group.

4.3.5 Effect of TGP on MDA content in the hippocampus of CUMS-treated rats

As shown in Figure 4.6, 5-week CUMS exposure significantly increased the content of MDA in the hippocampus of rats (94%), as compared to the control. TGP (80 and 160 mg/kg) treatment significantly decreased MDA content in the hippocampus of CUMS-treated rats (22% and 32%, respectively), as compared with the vehicle-treated and CUMS-treated rats.

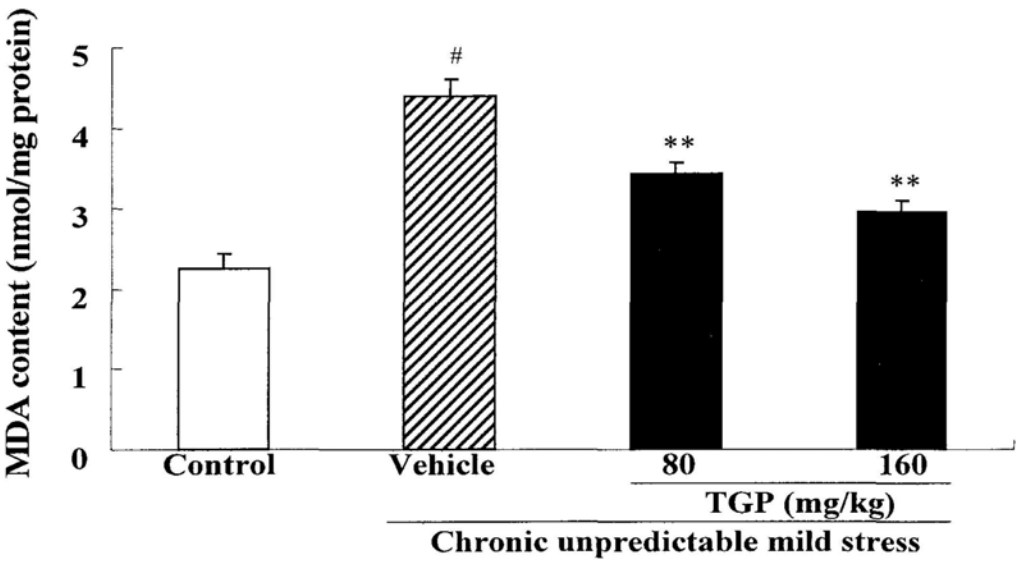


Figure 4.6 Effect of TGP on MDA content in the hippocampus of CUMS-treated rats. Values given are the mean ± SEMs (n = 8).
$^{\#}p$ <0.01 as compared to the control; $^{**}p$ <0.01 as compared with the vehicle-treated CUMS group.

4.3.6 Effect of TGP on GSH content in the hippocampus of CUMS-treated rats

Figure 4.7 shows the effect of TGP treatment on GSH content in the hippocampus of CUMS-treated rats. A 5-week CUMS exposure caused a significant decrease in the content of GSH in the hippocampus of rats (45%), as compared to the control. TGP (80 and 160 mg/kg) treatment significantly increased GSH content in the hippocampus of CUMS-treated rats (26% and 43%, respectively), as compared with the vehicle-treated and CUMS-treated rats.

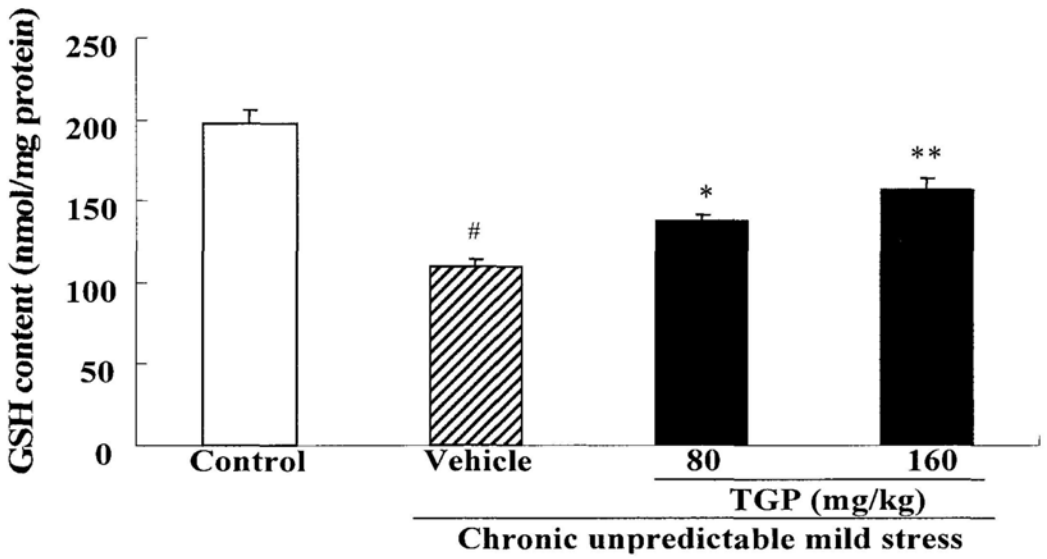


Figure 4.7 Effect of TGP on GSH content in the hippocampus of CUMS-treated rats. Values given are the mean ± SEMs (n = 8).
$^{\#}p$ <0.01 as compared to the control; $^{*}p$ <0.05, $^{**}p$ <0.01 as compared with the vehicle-treated CUMS group.

4.3.7 Effect of TGP on SOD activity in the hippocampus of CUMS-treated rats

Figure 4.8 shows the effect of TGP treatment on SOD activity in the hippocampus of CUMS-treated rats. A 5-week CUMS exposure caused a significant decrease in the antivity of SOD in the hippocampus of rats (35%), as compared to the control. TGP (80 and 160 mg/kg) treatment significantly increased SOD activity in the hippocampus of CUMS-treated rats (30% and 41%, respectively), as compared with the vehicle-treated and CUMS-treated rats.

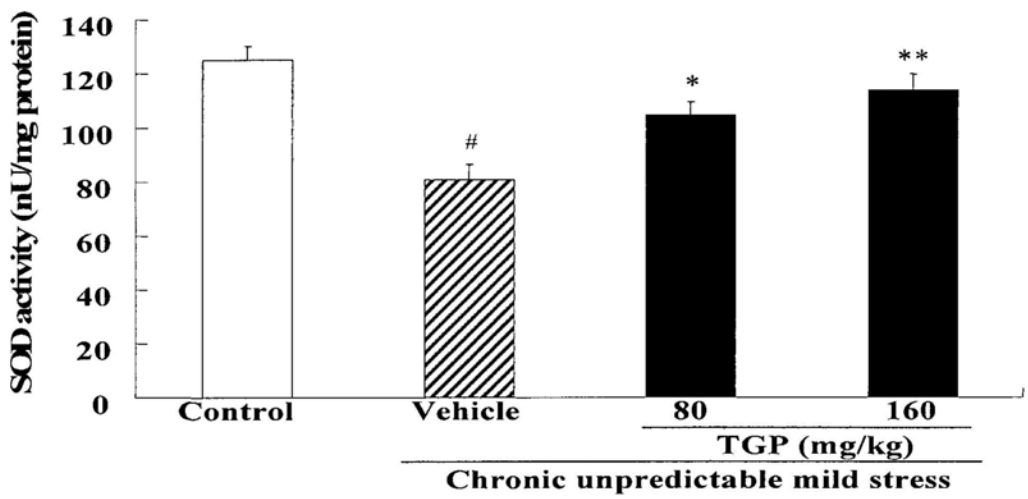


Figure 4.8 Effect of TGP on SOD activity in the hippocampus of CUMS-treated rats. Values given are the mean ± SEMs (n = 8).
$^{\#}p < 0.01$ as compared to the control; $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with the vehicle-treated CUMS group.

4.3.8 Effect of TGP on CAT activity in the hippocampus of CUMS-treated rats

Figure 4.9 shows the effect of TGP treatment on CAT activity in the hippocampus of CUMS-treated rats. A 5-week CUMS exposure significantly decreased the activity of CAT in the hippocampus of rats (29%), as compared to the control. TGP (160 mg/kg) treatment significantly increased CAT activity in the hippocampus of CUMS-treated rats (22%), as compared with the vehicle-treated and CUMS-treated rats.

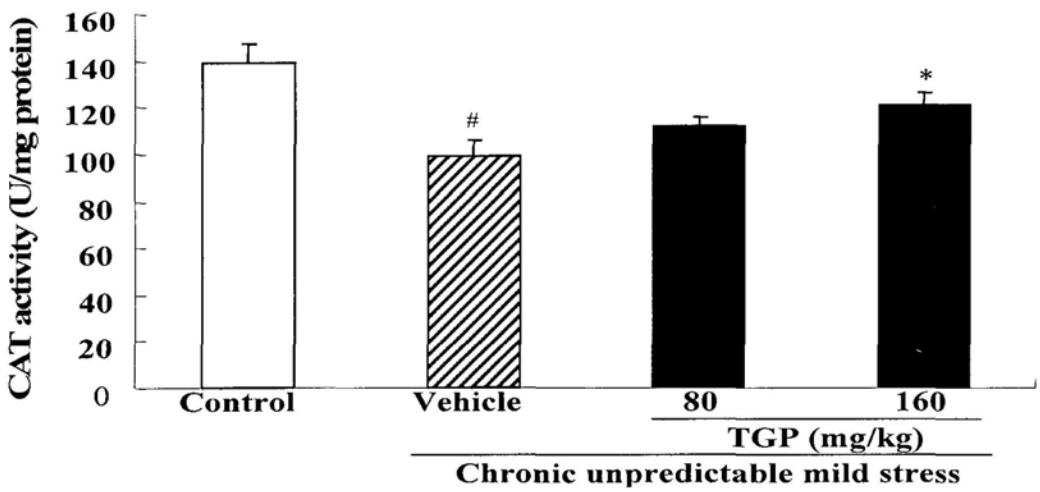


Figure 4.9 Effect of TGP on CAT activity in the hippocampus of CUMS-treated rats. Values given are the mean ± SEMs (n = 8).
$^{\#}p$ <0.01 as compared to the control; $^{*}p$ <0.05 as compared with the vehicle-treated CUMS group.

4.3.9 Effect of TGP on BDNF protein and mRNA levels in the hippocampus of CUMS-treated rats

Effects of TGP treatment on BDNF protein and mRNA levels in the hippocampus of CUMS-treated rats are showed in Figure 4.10A and Figure 4.10B respectively. A 5-week CUMS exposure significantly decreased the BDNF protein level in the hippocampus (43%), as compared to the control. TGP treatment at doses of 80 and 160 mg/kg significantly increased the BDNF protein level in the hippocampus of rat exposed to CUMS (29% and 65%, respectively), as compared to the vehicle-treated and CUMS-treated rats. BDNF mRNA level in the hippocampus was also found to be significantly decreased in CUMS-treated rats (53%), as compared to the control. TGP treatment at doses of 80 and 160 mg/kg significantly increased the BDNF mRNA level in the hippocampus of rats exposed to CUMS (48% and 78%, respectively), as compared to the vehicle-treated and CUMS-treated rats.

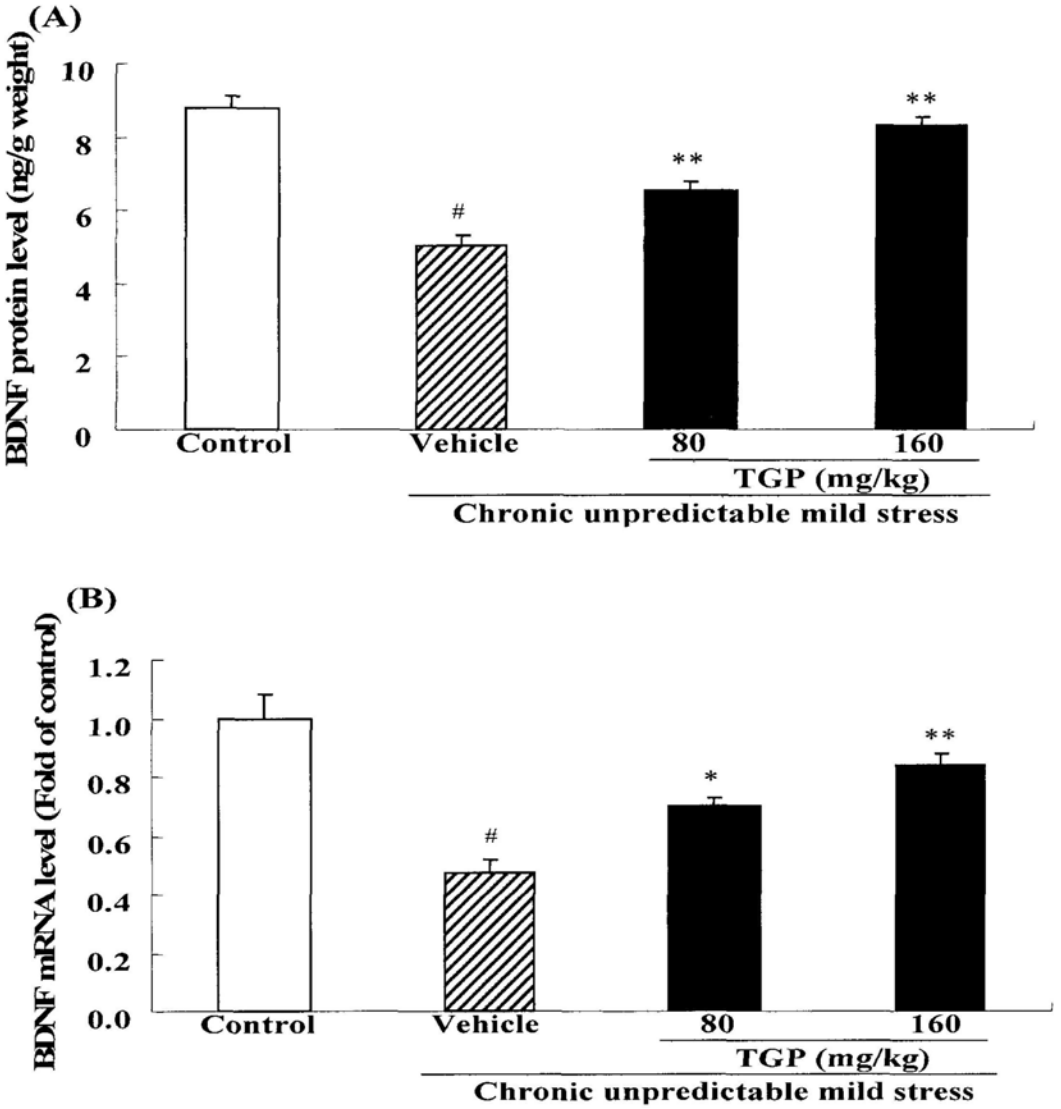


Figure 4.10 Effect of TGP on BDNF protein (A) and mRNA (B) levels in the hippocampus of CUMS-treated rats

Values given are the mean ± SEMs (n = 6).

$^{\#}p$ <0.01 as compared tothe control; $^{*}p$ <0.05, $^{**}p$ <0.01 as compared with the vehicle-treated CUMS group.

4.3.10 Effect of TGP on NGF protein and mRNA levels in the hippocampus of CUMS-treated rats

Effects of TGP treatment on NGF protein and mRNA levels in the hippocampus of CUMS-treated rats are showed in Figure 4.11A and Figure 4.11B respectively. There were no significant changes in the NGF protein and mRNA levels in the hippocampus among all rats.

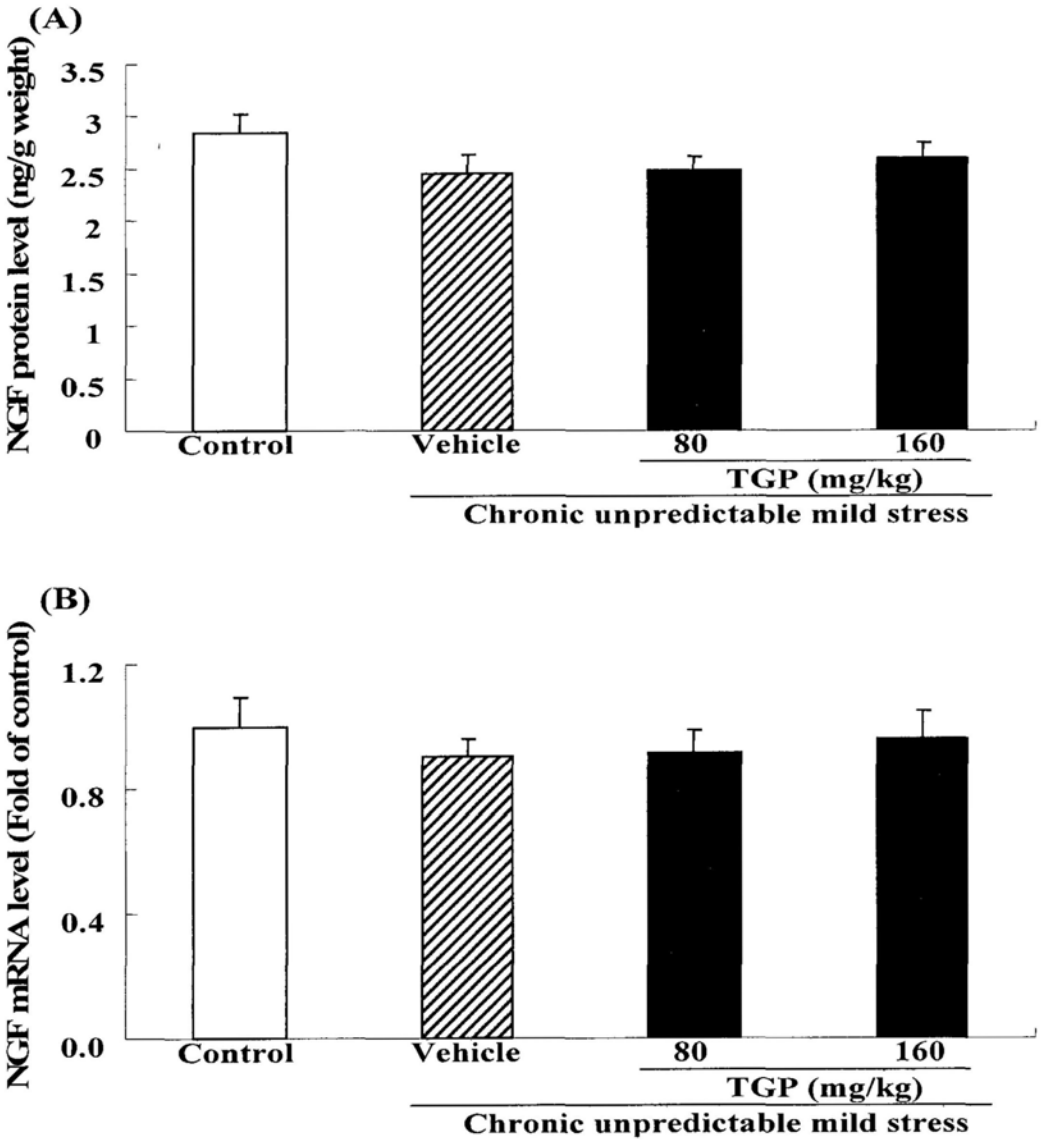


Figure 4.11 Effect of TGP on NGF protein (A) and mRNA (B) levels in the hippocampus of CUMS-treated rats.

Values given are the mean ± SEMs (n = 6).

4.3.11 Effects of TGP on NT-3 protein and mRNA levels in the hippocampus of CUMS-treated rats

Effects of TGP treatment on NT-3 protein and mRNA levels in the hippocampus of CUMS-treated rats are showed in Figure 4.12A and Figure 4.12B respectively. A 5-week CUMS exposure caused a significant decrease in the protein level of NT-3 in the hippocampus of rats (50%), as compared to the control. TGP treatment at doses of 80 and 160 mg/kg significantly increased the NT-3 protein level in the hippocampus of rat exposed to CUMS (53% and 77%, respectively), as compared to the vehicle-treated and CUMS-treated rats. NT-3 mRNA level in the hippocampus was also found to be significantly decreased in CUMS-treated rats (36%), as compared to the control. TGP treatment at doses of 80 and 160 mg/kg significantly increased the NT-3 mRNA level in the hippocampus of rats exposed to CUMS (28% and 41%, respectively), as compared to the vehicle-treated and CUMS-treated rats.

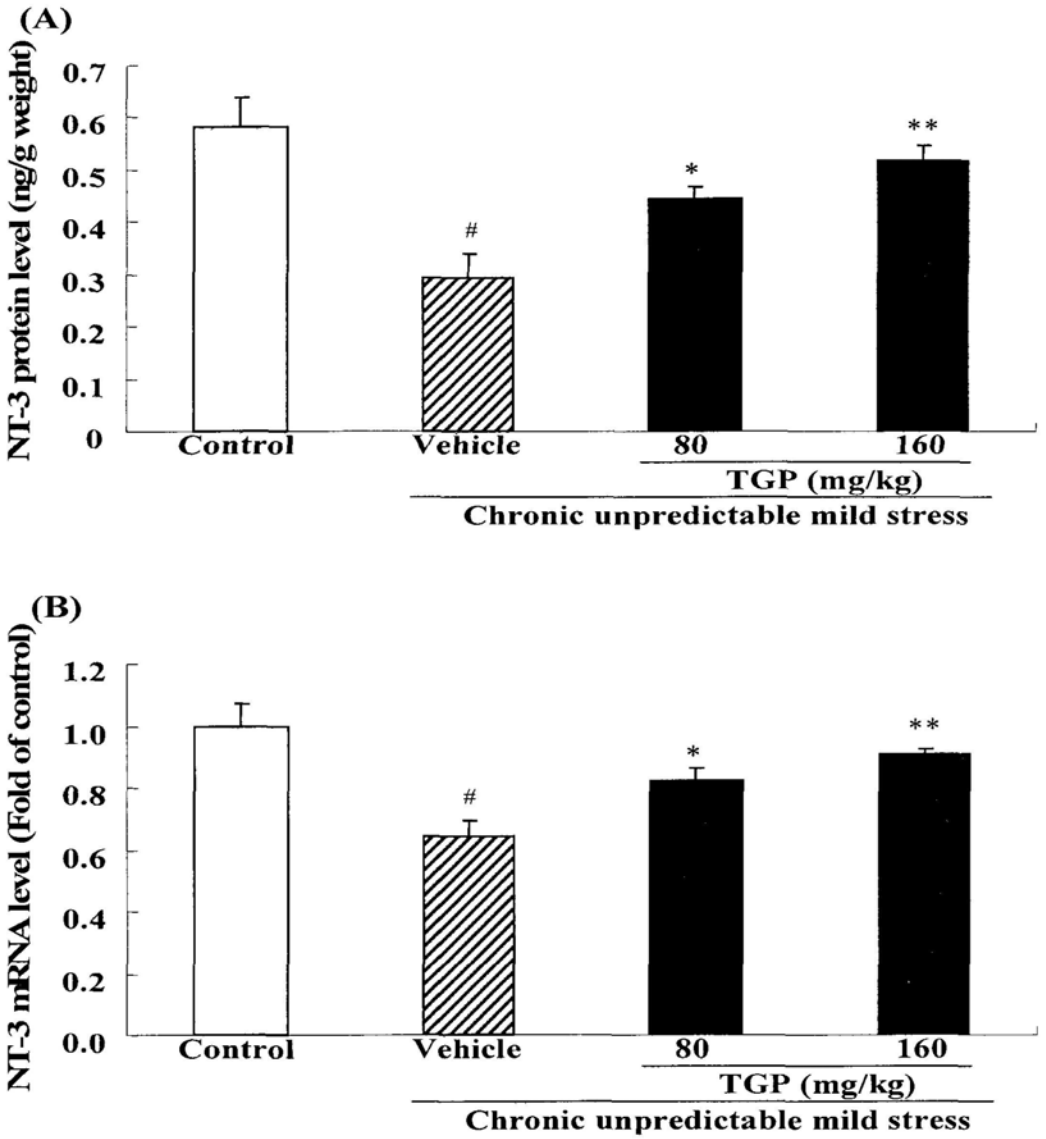


Figure 4.12 Effect of TGP on NT-3 protein (A) and mRNA (B) levels in the hippocampus of CUMS-treated rats

Values given are the mean ± SEMs (n = 6).

$^{\#}p < 0.01$ as compared to the control; $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with the vehicle-treated CUMS group.

4.4 Discussion

Chronic stress is a risk factor for major depression, and chronic stress paradigm in rodents is often used as an animal model of depression because it recapitulates a multitude of behavioral characteristics and biochemical states of depression in human (Willner, 1997 and 2005). In addition, chronic stress has also been shown to induce a significant decrease in the volume of hippocampus, and chronic antidepressant treatment was found to restore the normal volume of hippocampus (Czéh et al., 2001; van der Hart et al., 2002). The CA3 region of hippocampal neurons is the most vulnerable target to stress (Magariños and McEwen, 1995; Magariños et al., 1999; Zhang et al., 2003). Several studies showed that chronic stress could induce atrophy and destruction of the hippocampal CA3 pyramidal neurons in animals, while chronic treatment with antidepressants significantly reversed these changes (Magariños and McEwen, 1995; Magariños et al., 1999; Zhang et al., 2003; Takuma et al., 2007). Consistent results were observed in this study. A 5-week CUMS caused hippocampal neuronal damage in rats, as indicated by a significant decrease in the number of pyramidal neuronal cells in the hippocampal CA3 region. Long-term treatment with TGP markedly inhibited the decrease of neuronal cells in the hippocampal CA3 region of CUMS-treated rats, suggesting that TGP produced

neuroprotective effects on the hippocampus in CUMS-treated rats.

The hypothalamic-pituitary-adrenal (HPA) axis plays a key role in eliciting physiological responses to various stressful stimuli (Pan et al., 2006). It is well known that HPA axis is activated in response to stress, resulting in an increase of circulating glucocorticoids such as corticosterone in rodents or cortisol in primates. Sustained activation of the HPA axis is associated with an abnormally high blood glucocorticoid level, which may eventually lead to pathological damages in the hippocampal neurons (Johnson et al., 2006; Murray et al., 2008). In this connection, it has been reported that therapeutic actions of antidepressants are produced through a modulation of HPA axis in the depressive patients (Nikisch et al., 2005; Himmerich et al., 2007). Thus, the restoration of a normal functional status of HPA axis may be critically involved in the therapeutic intervention of depression (Pan et al., 2006). The present finding of CUMS-induced hyperactivity of HPA axis, as indicated by an increased serum corticosterone level, is corroborated by observations from other studies (Pan et al., 2006; Xu et al., 2006; Li et al., 2007b; Sheikh et al., 2007). TGP treatment significantly decreased serum corticosterone level in CUMS-treated rats, suggesting that TGP could inhibit the hyperactivity of HPA axis in depressive rats.

Elevated blood level of glucocorticoid exerts feedback regulation on the hyperactive HPA axis (Xu et al., 2006; Rybnikova et al., 2007), wherein two distinct intracellular receptor subtypes, namely, MR and GR, are involved (Reul and de Kloet, 1985). Hippocampus, which is a target for stress hormones, contains high levels of MR and GR (Budziszewska, 2002; Racca et al., 2005). MR has a high affinity for endogenous corticosteroids, and is believed to play a role in the regulation of circadian fluctuations in these hormones (Raone et al., 2007; Zhou et al., 2008). In contrast, GR has a lower affinity for endogenous corticosteroids (Raone et al., 2007), and is believed to regulate stress responses, particularly when the glucocorticoid level is high (Neigh and Nemeroff, 2006; Xu et al., 2006; Raone et al., 2007; Zhou et al., 2008). With regard to this, it has been demonstrated that the GR mRNA level in the hippocampus was decreased in depressive animals, and long-term administration of antidepressants could attenuate this change (Pariante and Miller, 2001; Xu et al., 2006; Raone et al., 2007). Consistent to these observations, the present results showed that the GR mRNA level in the hippocampus was significantly decreased in CUMS-treated rats, with the MR mRNA level being unaffected. TGP treatment, which was found to increase the GR mRNA level in the hippocampus of CUMS-treated rats, may be able to modulate the feedback regulation on stress response.

Oxidative stress is defined as a disturbance in the balance between the production of ROS level and antioxidant defenses (Yazdanparast et al., 2008). Previous studies have demonstrated that chronic stress caused a significant increase in the production of ROS level (Lucca et al., 2009). Excessive ROS level can cause damages to the major macromolecules in cells, including lipids, proteins, and nucleic acids (Niebrój-Dobosz et al., 2004; Zhao et al., 2008), culminating in neuronal dysfunction and depression. MDA, a by-product of lipid peroxidation, is produced under oxidative stress. It reflects the oxidative damages of the plasma membrane and resultant thiobarbituric acid reactive substances, which are proportional to lipid peroxidation and oxidant stress (Xiao et al., 2008). It has been reported that MDA level was significantly increased in the hippocampus of rats exposed to chronic stress, which could be reversed by antidepressants treatment (Zafir and Banu, 2007; Lucca et al., 2009). The present study demonstrated that CUMS caused a significant increase in the levels of intracellular ROS and MDA in the hippocampus, while long-term treatment with TGP significantly reversed these changes. On the other hand, biological antioxidants are natural compounds which can prevent the uncontrolled formation of free radicals and activated oxygen species, or inhibit their reaction with biological structures (Yazdanparast et al., 2008). These compounds include antioxidative enzymes, such as SOD and CAT, and non-enzymatic antioxidants, such as

GSH (Yazdanparast et al., 2008). SOD and CAT have been shown to directly catalyze the transformation of peroxides and superoxides to nontoxic species (Griendling and Ushio-Fukai, 2000). GSH, the most abundant natural antioxidant, also plays a critical role in the anti-ROS defense system (Mueller et al., 2001). The efficiency of this antioxidant defense system is apparently weakened in depressive disorders (Lukash et al., 2002), resulting in an ineffective scavenging of free radicals which may lead to tissue damages (Niebrój-Dobosz et al., 2004; Zhao et al., 2008). Chronic stress has also been shown to cause an impairment of antioxidant defenses in rat hippocampus, and antidepressant treatment was found to restore the normal level of antioxidant defenses (Zafir and Banu, 2007; Lucca et al., 2009). Consistent to such hypothesis, the present result showed that GSH level, SOD and CAT activities were all significantly decreased in the hippocampus of CUMS-treated rats as compared to untreated controls. Chronic treatment with TGP significantly increased GSH level, SOD and CAT activities in the hippocampus of CUMS-treated rats.

Neurotrophins are a family of proteins which modulate neuronal plasticity, inhibit cell death cascades and increase cell survival proteins that are responsible for proliferation and maintenance of central nervous system neurons (Huang et al., 2001; Blum and Konnerth,

2005). Thus, increasing neurotrophins contents to ameliorate the neuronal atrophy and destruction in the hippocampus may be an important therapeutic intervention of depression (Altar. 1999; Castrén and Rantamäki, 2008). BDNF, NGF and NT-3 are the most abundant neurotrophic factors in the central nervous system. Interestingly, in recent years, it has also been demonstrated that abnormal BDNF, NGF and NT-3 expression were closely associated with depression (Nestler et al., 2002; Angelucci et al., 2003; von Richthofen et al., 2003; Lang et al., 2004a and b; Dwivedi et al., 2005; Karege et al., 2002 and 2005; Duman and Monteggia, 2006; Topic et al., 2008; Huston et al.. 2009).

It has been found that BDNF content was decreased in the hippocampus from suicide victims with major depression (Karege et al., 2005), whereas brain infusion of BDNF produced antidepressant-like action in animals (Siuciak et al., 1997). Clinical studies have shown that a decrease in serum BDNF level was observed in depressive patients (Karege et al., 2002 and 2005a; Aydemir et al., 2006; Aydemir et al., 2007), and antidepressant treatment could restore the normal level of BDNF (Shimizu et al., 2003; Başterzi et al., 2009). BDNF content was found to be decreased in depressive rodents as well, which could be reversed by long term antidepressant treatment (Xu et al., 2006; Li et al., 2007b; Zheng 2006). Moreover, Monteggia et al. (2007) showed that conditional

BDNF knockout mice displayed an enhancement of depression-like behavior. Ibarguen-Vargas and colleagues (2009) have demonstrated that a deficit in BDNF can dampen the effects of antidepressant in mice exposed to unpredictable chronic mild stress. These data suggest that BDNF plays an important role in the molecular pathophysiology of depression and up-regulation of BDNF expression may contribute to the action of antidepressants (Shimizu et al., 2003; Başterzi et al., 2009). Consistent to such hypothesis, in the present study, a 5-week CUMS exposure was found to decrease BDNF protein and mRNA levels in the hippocampus of rats, and TGP treatment reversed the CUMS-induced changes. Furthermore, the present study revealed that a 5-week CUMS exposure significantly decreased NT-3 protein and mRNA levels in the hippocampus of rats, while TGP significantly increased NT-3 protein and mRNA levels. Indeed, it has been well demonstrated that NT-3 is also involved in the pathogenesis of depression. A postmortem study in suicide victims has shown decreased NT-3 protein levels in the hippocampus (Karege et al., 2005). A recent clinical study has also shown a significant decrease in NT-3 expression in depressive patients (Otsuki et al., 2008). The NT-3 protein level was also found to decrease in the hippocampus of depressive rats, whereas chronic treatment with antidepressant increased the NT-3 protein level in the hippocampus (Chen et al., 2008; Topic et al., 2008). In addition, the present study demonstrated that a

5-week CUMS did not alter NGF protein and mRNA levels in the hippocampus of rats as compared to untreated controls. So far, contradictory data have been reported on hippocampal NGF expression in different animal models of stress. In rodents, NGF mRNA level was increased in the hippocampus after one or repeated episodes of cold stress (Foreman et al., 1993), but remained unchanged after social isolation and electric foot shocks (Spillantini et al., 1989; Schulte-Herbrüggen et al., 2006), whereas hippocampal NGF content was reduced after chronic psychosocial stress (Alfonso et al., 2004). The reason for these conflicting results on NGF expression in the hippocampus might be due to the variety in animal species and the intensity of the stressors.

In conclusion, long-term TGP treatment was found to produce neuroprotective effects on the hippocampus of CUMS-treated rats. This effect was associated with the attenuation of HPA axis hyperactivation, and the inhibition of oxidative stress, and the up-regulation of BDNF and NT-3 levels in the hippocampus.

Chapter 5

Neuroprotective effects of total glycosides of peony against corticosterone-induced cell death in PC12 cells and its possible mechanisms

5.1 Introduction

In recent years, a substantial amount of preclinical and clinical data have supported the notion that neuronal atrophy/destruction in the hippocampus is involved in the pathogenesis of depression (Manji and Duman, 2001; Fuchs et al., 2004). Several putative mechanisms have been implicated. One of them is the hyperactivation of hypothalamic-pituitary-adrenal (HPA) axis, with the latter being characterized by elevated levels of circulating glucocorticoids in blood (Magariños and McEwen, 1995; Sapolsky, 2000; Aihara et al., 2007; Watson and Mackin, 2007; Murray et al., 2008). It is well known that the HPA axis is activated in response to stress, which results in the increased concentration of glucocorticoid in circulating blood. Under normal condition, blood glucocorticoid level is tightly regulated by a negative feedback mechanism. However, it has been reported that high concentration of blood glucocorticoid was maintained in patients suffered from depression due to the dysfunction of feedback mechanism (Johnson

et al., 2006; Watson and Mackin, 2007). High glucocorticoid level can cause pathological damages in hippocampal neurons (Magariños and McEwen, 1995; Sapolsky, 2000; Johnson et al., 2006; Murray et al., 2008).

The rat adrenal pheochromocytoma (PC12) cells, possessing typical features of neurons and expressing high level of glucocorticoid receptors (Anderson and Michelsohn, 1989), have been widely used as an neuronal cell line in a variety of studies. Treatment of the PC12 cell line with high concentrations of glucocorticoid to simulate hippocampal nuronal damage in depressive disorder, has been used as an *in vitro* experimental model of depression (Gao et al., 2008; Li et al., 2003b and 2003c; Li et al., 2004; Zhu et al., 2006b). Different types of classical antidepressants have been shown to protect against cytotoxicity induced by glucocorticoid in PC12 cells (Li et al., 2003c), suggesting that cytoprotection against glucocorticoid-induced neurotoxicity may represent one of the action pathways of antidepressants.

Previous studies (Chapter 2) have demonstrated the antidepressant-like effect of total glycosides of peony (TGP) using forced swim and tail suspension tests. The antidepressive effect of TGP was also observed in rats exposed to chronic unpredictable

mild stress (Chapter 3). However, the mechanism by which TGP exerts its antidepressant-like effect is not fully understood. The present study aims to examine the neuroprotective effects of TGP on cultured PC12 cells exposed to corticosterone, a principal glucocorticoid. In order to investigate the mechanism(s) underlying the neuroprotective effects of TGP, intracellular reactive oxygen species (ROS) level, reduced glutathione (GSH) content, malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, catalase (CAT) activity, and neurotrophins such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin-3 (NT-3) protein and mRNA levels were measured in corticosterone-treated PC12 cells, with or without TGP treatment.

5.2 Materials and methods

5.2.1 Drugs and chemical reagents

TGP was supplied by Ningbo Liwah Pharmaceutical Co., Ltd. (Zhejiang, China). 2',7'-Dichlorofluorescin diacetate (DCFH-DA) was obtained from Invitrogen (Carlsbad, CA, USA). 5,5-Dithiobis-2-nitrobenzoic acid, corticosterone and DMEM culture medium were obtained from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum,

heat-inactivated horse serum, penicillin and streptomycin were purchased from Gibco (Grand Island, NY, USA). All other chemicals and reagents were of analytical grade.

5.2.2 Cell culture and treatment

PC12 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). They were maintained in DMEM medium supplemented with penicillin (100 unit/mL), streptomycin (100 μg/mL), 6% fetal bovine serum and 6% horse serum at 37 °C in humidified atmosphere of 95% air and 5% CO_2. The experimental design contained five treatment groups: non-treated control, 200 μM corticosterone and 200 μM corticosterone plus TGP (1, 5 or 10 mg/L). The cells were seeded onto 96-well culture plate at 2×10^4 cells/well, unless otherwise specified. The cells were stabilized at 37 °C for 48 hours. Then they were cultured in serum-free medium and incubated with corresponding drugs for another 48 hours.

5.2.3 Cell viability assay

Cell viability was measured by a CellTiter 96® AQ_{ueous} One Solution Cell Proliferation (MTS) Assay (Promega, Madison, WI, USA). Briefly, the cells were washed with D-Hanks solution after drug treatment. Then 100 μL of serum-free medium and 20 μL of MTS were added into each well. The cells were incubated at 37 °C for 1 hour. The quantity of formazan product, which is directly proportional to the number of living cells, was measured by a microplate reader at 490 nm. Cell viability was expressed as a percentage of non-treated control.

5.2.4 Trypan blue exclusion assay

Cell viability was also measured by trypan blue exclusion assay according to the method of Cho et al. (2009). Briefly, PC12 cells were seeded in 6-well culture plates at a density of 2×10^6 cells/well. At the end of drug treatment, the cells were suspended. After centrifugation at 600 g for 6 min, cells were resuspended in 200 μL of phosphate-buffered saline (PBS). The total cell suspension was mixed with 200 μL of 0.4% trypan blue staining solution for 5 min at room temperature. The cells were then loaded into a

hemocytometer, and those exhibiting dye uptake were counted under a microscope. The percentage of stained cells was counted by scoring 150 cells. Cell viability was expressed as a percentage of non-treated control.

5.2.5 Cytotoxicity assay

Lactate dehydrogenase (LDH) release was measured as an in vitro marker for cellular toxicity. LDH activity was measured using a LDH diagnostic kit (STANBIO Laboratory, USA) according to the manufacturer's protocol. Briefly, PC12 cells were seeded in 24-well culture plates at a density of 1×10^5 cells/well. At the end of drug treatment, the medium was collected. Subsequently, 100 μL of medium was added to a polystyrene cuvet containing 1000 μL of LDH reagent (50 mM lactate, 7 mM NAD^+ in 0.05% sodium azide buffer pH = 8.9). The cuvet was placed immediately into the spectrophotometer, maintained at 30 °C. After stabilization for 1 min, absorbance at 340 nm was recorded at 1 min intervals for 3 min. The change in absorbance was then expressed in concentration units per liter. To determine intra-cellular LDH activity, the cells were washed with D-Hanks solution, then scraped from the plates into 500 μL ice cold PBS (0.1M, containing 0.05mM EDTA) and homogenized. The homogenate was centrifuged (4000 *g*)

at 4 °C for 30 min. The resulting supernatants were collected for assay of the LDH activity. LDH leakage was expressed as the percentage (%) of the total LDH activity (LDH in the medium+ LDH in the cell), according to the equation %LDH released = (LDH activity in the medium/total LDH activity) ×100.

5.2.6 Measurement of intracellular ROS level

The Intracellular ROS level was measured using the 2',7'-dichlorofluorescin diacetate (DCFH-DA) method (Yokozawa et al., 2007). DCFH-DA is a nonfluorescent compound, and it can be enzymatically converted to highly fluorescent compound, DCF, in the presence of ROS. Briefly, the cells were washed with D-Hanks solution after drug treatment. Then the cells were incubated with DCFH-DA at a final concentration of 10 μM for 30 min at 37 °C in darkness. After the cells were washed twice with D-Hanks solution to remove the extracellular DCFH-DA, the fluorescence intensity of DCF was measured in a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The ROS level was normalized to the protein concentration of each sample and expressed as a percentage of non-treated control.

5.2.7 Measurement of MDA content

MDA content was measured as previously described (Ohkawa et al., 1979) using thiobarbituric acid. Briefly, PC12 cells were seeded onto 6-well culture plate at a 2×10^6 cells /well. At the end of drug treatment, the cells were washed with D-Hanks solution, then scraped from the plates into 500 μL ice cold PBS (0.1 M, containing 0.05 mM EDTA) and homogenized. The homogenate was centrifuged (4000 g) at 4 °C for 30 min. The resulting supernatants were stored at –80 °C until assay. An aliquot (100 μL) of supernatant was mixed with 1500 μL acetic acid (20% [v/v], pH 3.5), 1500 μL thiobarbituric acid (0.8%, w/v) and 200 μL sodium dodecyl sulfate (8%, w/v). Each reaction mixture was heated at 95 °C for 60min and cooled to room temperature. Next, 5000 μL of n-butanol was added. After mixing and centrifugation at 3000 g for 10 min, the organic layer was collected and the absorbance measured at 532 nm. The MDA level was normalized to the protein concentration of each sample and expressed as a percentage of non-treated control.

5.2.8 Measurement of GSH content

GSH content was measured as previously described (Gulati et al., 2007). Briefly, PC12 cells were seeded onto 100-mm^2 dish at 5 $\times 10^6$ cells /dish. At the end of drug treatment, the cells were washed with D-Hanks solution, then scraped from the dishes into 500 μL ice cold PBS (0.1 M, containing 0.05 mM EDTA) and homogenized. The homogenate was centrifuged (4000 g) at 4 °C for 30 min. The resulting supernatants were stored at –80 °C until assay. An aliquot (100 μL) of supernatant was mixed with 200 μL trichloroacetic acid (25%, v/v) and 200 μL saline. The mixture was centrifuged (3000 g) for 10 min at 4 °C. 200 μL of supernatant was mixed with 1000 μL phosphate buffer (100 mM, pH 8.0) and 50 μl 5,5-Dithiobis-2-nitrobenzoic acid (3 mM). The solution was held at room temperature for 5 min and the absorbance measured at 412 nm. The GSH level was normalized to the protein concentration of each sample and expressed as a percentage of non-treated control.

5.2.9 Measurement of SOD activity

SOD activity was measured using a SOD activity assay kit (BioVision, Mountain View, CA) according to the manufacturer's protocol. Briefly, PC12 cells were seeded onto 100-mm^2 dish at 5 $\times 10^6$ cells /dish. At the end of drug treatment, the cells were washed with D-Hanks solution, then scraped from the dishes into 500 μL ice cold PBS (0.1 M, containing 0.05 mM EDTA) and homogenized. The homogenate was centrifuged (4000 g) at 4 °C for 30 min. The resulting supernatants were stored at –80 °C until assay. 20 μl of supernatant was added with 200 μL of water soluble tetrazolium working solution and 20 μL of enzyme working solution into a 96-well plate. After incubating the plate at 37 °C for 20 min, the absorbance at 450 nm was read using a microplate reader. The SOD activity was normalized to the protein concentration of each sample and expressed as a percentage of non-treated control.

5.2.10 Measurement of CAT activity

CAT activity was measured according to the method of Shen et al. (2009). Briefly, PC12 cells were seeded onto 100-mm^2 dish at 5 $\times 10^6$ cells /dish. At the end of drug treatment,

the cells were washed with D-Hanks solution, then scraped from the dishes into 500 μL ice cold PBS (0.1 M, containing 0.05 mM EDTA) and homogenized. The homogenate was centrifuged (4000 g) at 4 °C for 30 min. The resulting supernatants were stored at –80 °C until assay. 50 μl of supernatant was mixed with 1950 μL phosphate buffer (0.05 M, pH 7.0) and 1000 μL hydrogen peroxide (0.019 M). The activity of CAT was estimated by the decrease of absorbency at 240 nm for 2 min. The CAT activity was normalized to the protein concentration of each sample and expressed as a percentage of non-treated control.

5.2.11 Measurement of BDNF, NGF and NT-3 mRNA levels

BDNF, NGF and NT-3 mRNA levels were measured using TaqMan real-time reverse transcription-PCR analysis. Briefly, PC12 cells were seeded onto 100-mm^2 dish at 5 $\times 10^6$ cells /dish. The cells were washed with D-Hanks solution after drug treatment. Total RNA from the cells was isolated with TRIzol Reagent (Gibco, Grand Island, NY) according to the manufacturer's protocol. Concentrations of extracted RNA were calculated from the absorbance at 260 nm. The quality of RNA was assessed by absorbances at 260 nm and 280 nm, with the ratio of A_{260} to A_{280} ranging from 1.9 to 2.1 being acceptable. Total RNA (1.5 μg) was transcribed using a High Capacity cDNA

Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's protocol. Assays-on-Demand primers for BDNF (Rn02531967_s1), NGF (Rn01533872_m1), NT-3 (Rn00579280_m1) and β-actin (Rn00667869_m1) were purchased from Applied Biosystems, Inc (Foster City, CA). Real-time quantitative PCR analysis was performed with a TaqMan® Fast Universal PCR Master Mix Kit (Applied Biosystems, Inc., Foster City, CA) by using StepOnePlus™ Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA) with the following profile: 2 min hold at 50 °C, 10 min hold at 95 °C, followed by 60 cycles of 15 sec at 95 °C and 1 min at 60 °C. Sequence Detection Software 2.0 (Applied Biosystems, Inc., Foster City, CA) was used for data analysis. The relative expression of BDNF, NGF and NT-3 mRNA was normalized to the amount of β-actin in the same cDNA using the relative quantification method ($2^{-\Delta\Delta CT}$ method) as described by the manufacturer.

5.2.12 Measurement of BDNF, NGF and NT-3 protein levels

PC12 cells were seeded onto 100-mm^2 dish at 5 $\times 10^6$ cells /dish. At the end of drug treatment, the cells were washed with D-Hanks, then scraped from the plates into 500 μL ice cold PBS (0.1 M, containing 0.05 mM EDTA) and homogenized. The homogenate

was centrifuged at 4 °C at 10,000 g for 30 min. The resulting supernatants were used for BDNF, NGF and NT-3 assays.

Protein levels of BDNF were measured using a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kit (Chemicon International, Temecula, CA) according to the manufacturer's instructions. Briefly, samples and serial dilutions of BDNF standard (0-500 pg/mL) were applied in duplicate into 96-well immunoplates precoated with rabbit anti-human BDNF antibody and incubated on a shaker overnight at 4 °C. After washing four times, biotinylated mouse anti-human BDNF antibody was added and incubated for 3 h at room temperature. Then streptavidin-HRP conjugate solution was added and incubated at room temperature for 1 hour after washing. TMB/E substrate was added and incubated at room temperature for 15 minutes. The reaction was stopped with 1 M hydrochloric acid and absorbance recorded at 450 nm immediately. BDNF concentration was determined from the regression line for the BDNF standard curve incubated under similar conditions. The detection limit of the assay is ~ 7.8 pg/mL, and the cross-reactivity with other related neurotrophic factors (NGF, Neurotrophin-3 and Neurotrophin-4) was considered nil. BDNF concentration was normalized to the protein concentration of each sample and expressed as a percentage of non-treated control.

Protein levels of NGF were measured using a two-site ELISA kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 96-well immunoplates were coated with anti-NGF pAb (capture Ab). After an overnight incubation at 4 °C, the plate was blocked with Block and Sample Buffer for 1 h. Then samples and serial dilutions of NGF standard (0-250 pg/mL) were added in duplicate after washing and then shaken for 6 h at room temperature. After three washes, the detection antibody anti-NGF mAb was added and incubated overnight at 4 °C. After the incubation of anti-rat IgG HRP-conjugate for 2.5 h at room temperature and with orbital shaking, the substrate TMB one solution as added. The reaction was stopped with 1 M hydrochloric acid and absorbance recorded at 450 nm within 30 minutes. NGF concentrations were determined, from the regression line for the NGF standard incubated under similar conditions. The detection limit of the assay is ~ 7.8 pg/mL, and the cross-reactivity with other related neurotrophic factors (BDNF, Neurotrophin-3 and Neurotrophin-4) was less than 3%. NGF concentration was normalized to the protein concentration of each sample and expressed as a percentage of non-treated control.

Protein levels of NT-3 were measured using a two-site ELISA kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 96-well immunoplates were

coated with anti-NT-3 pAb (capture Ab). After an overnight incubation at 4 °C, the plate was blocked with Block and Sample Buffer for 1 h. Then samples and serial dilutions of NGF standard (0-300 pg/mL) were added after washing and then shaken for 6 h at room temperature. After three washes, the detection antibody anti-NT-3 mAb was added and incubated overnight at 4 °C. After the incubation of anti-rat IgG HRP-conjugate for 2.5 h at room temperature and with orbital shaking, the substrate TMB one solution as added. The reaction was stopped with 1 M hydrochloric acid and absorbance recorded at 450 nm. NT-3 concentrations were determined, from the regression line for the NT-3 standard incubated under similar conditions. The detection limit of the assay is ~ 9.4 pg/mL, and the cross-reactivity with other related neurotrophic factors (BDNF, NGF and Neurotrophin-4) was less than 3%. NT-3 concentration was normalized to the protein concentration of each sample and expressed as a percentage of non-treated control.

5.2.13 Measurement of protein concentration

The protein content was measured according to the method of Lowry (Lowry et al., 1951) using bovine serum albumin as standard.

5.2.14 Statistical analysis

Data were expressed as mean ± SEM. The GraphPad Prism software (version 4.0) was used to perform the statistics (GraphPad Software, Inc., San Diego, CA). One-way analysis of variance (ANOVA) followed by Dunnett's test was used to test the differences between the groups. Difference was considered statistically significant when the value $p < 0.05$.

5.3 Results

5.3.1 Effect of TGP on the cell viability in corticosterone-treated PC12 cells

According to the results of the MTS assay (Figure 5.1A), the exposure to corticosterone at concentration of 200 μM for 48 h caused a significant decrease in the cell viability (61%), as compared to the control. Co-treatment with TGP (1, 5 and 10 mg/L) significantly increased the cell viability in corticosterone-treated PC12 cells (17%, 23% and 43% respectively), as compared to the corticosterone group. In line with our MTS data, the results of the trypan blue assay revealed that TGP exerted protective effects against corticosterone-induced neuronal cell death in a dose-dependent manner (Figure 5.1B). Compared to the control group, the exposure to corticosterone at concentration of 200 μM for 48 h caused a significant decrease in the cell viability (70%). Co-treatment with TGP (1, 5 and 10 mg/L) significantly increased the cell viability in corticosterone-treated PC12 cells (26%, 48% and 62% respectively), as compared to the corticosterone group.

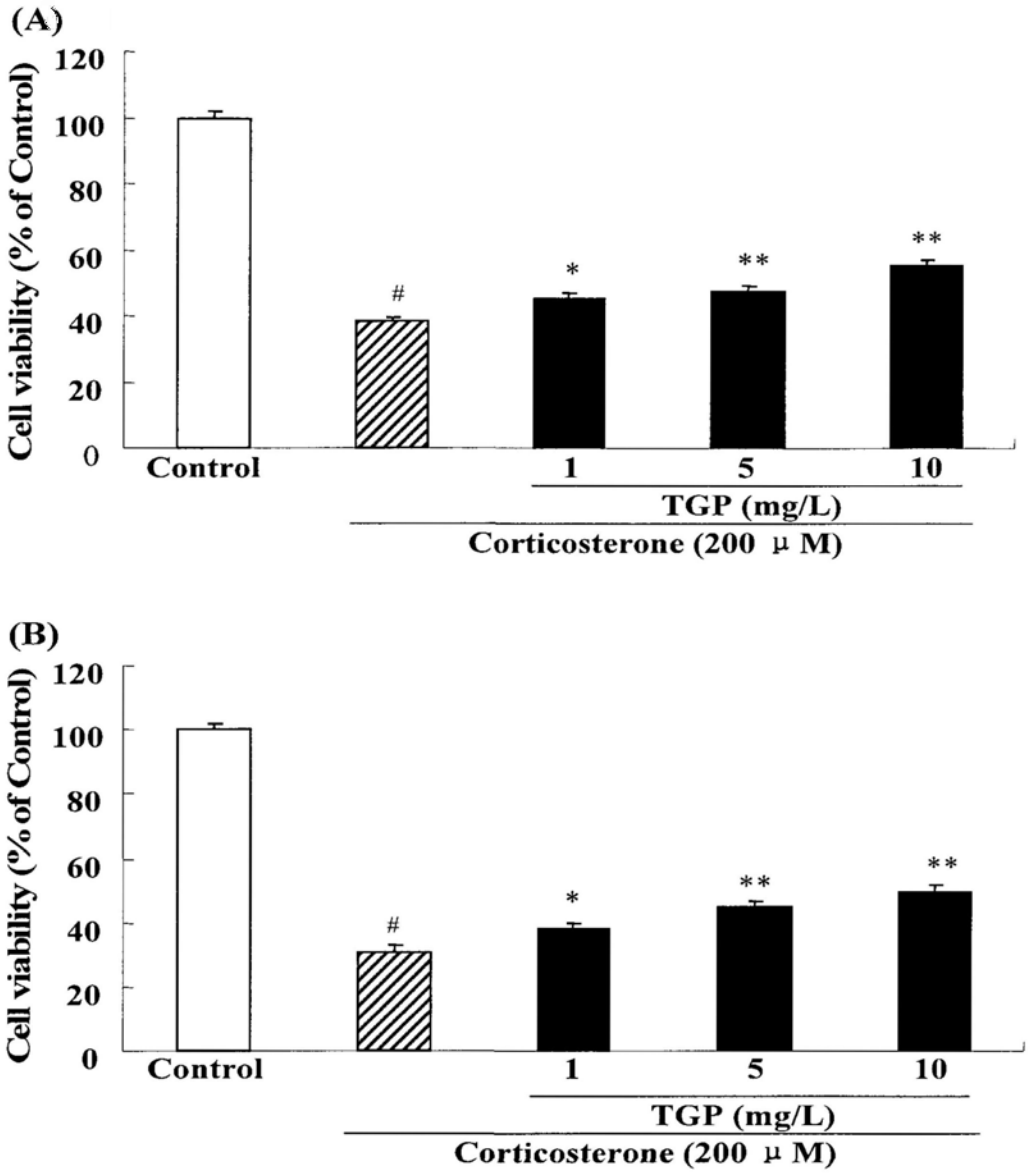


Figure 5.1 Effect of TGP on the cell viability in corticosterone-treated PC12 cells. Cell viability was determined by the MTS reduction assay (A) and the trypan blue exclusion assay (B).

Values given are the mean ± SEMs (n = 6-7).

$^{\#}p < 0.01$ as compared to the control; $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to the corticosterone group.

5.3.2 Effect of TGP on the LDH leakage in corticosterone-treated PC12 cells

To further investigate the protective effects of TGP, the release of LDH test was performed. As shown in Figure 5.2, the exposure to corticosterone at concentration of 200 μM for 48 h caused a significant increase in the LDH leakage (513%), as compared to the control. Co-treatment with TGP (1, 5 and 10 mg/L) significantly increased the cell viability in corticosterone-treated PC12 cells (18%, 32% and 40% respectively), as compared to the corticosterone group.

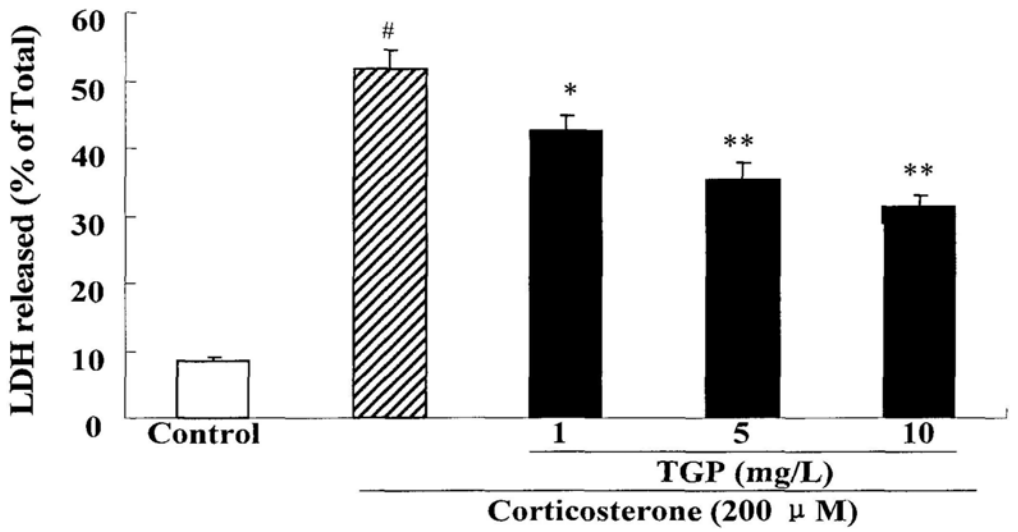


Figure 5.2 Effect of TGP on the LDH leakage in corticosterone-treated PC12 cells. Values given are the mean ± SEMs (n = 6).
$^{\#}p < 0.01$ as compared to the control; $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to the corticosterone group.

5.3.3 Effect of TGP on intracellular ROS level in corticosterone-treated PC12 cells

As shown in Figure 5.3, treating PC12 cells with corticosterone at concentration of 200 μM for 48 h caused a significant increase in the intracellular level of ROS (95%), as compared to the control. Co-treatment with TGP (1, 5 and 10 mg/L) significantly decreased intracellular ROS level in corticosterone-treated PC12 cells (18%, 24% and 34% respectively), as compared to the corticosterone group.

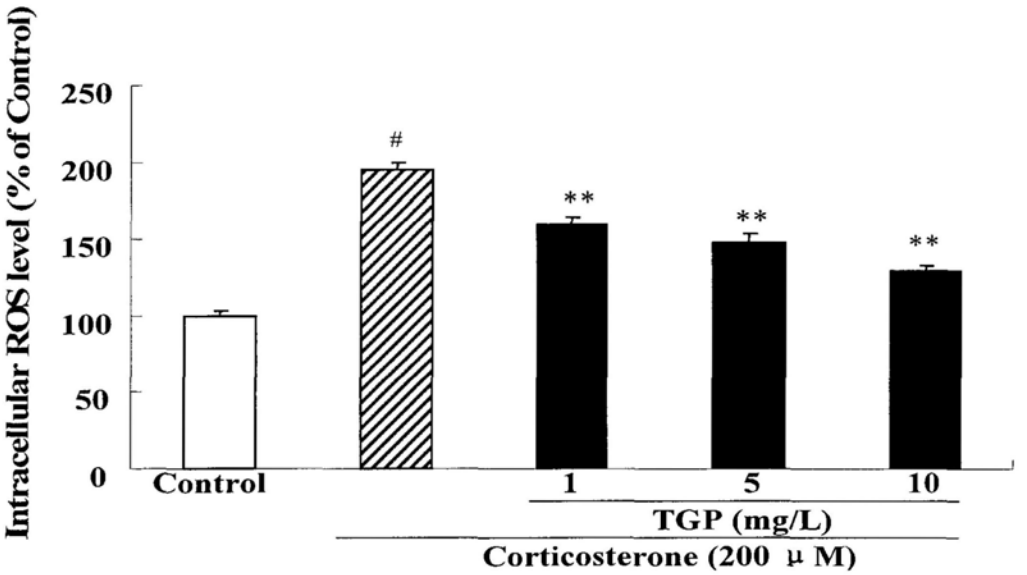


Figure 5.3 Effect of TGP on intracellular ROS level in corticosterone-treated PC12 cells. Values given are the mean ± SEMs (n = 6).

$^{\#}$ p <0.01 as compared to the control; ** p <0.01 as compared to the corticosterone group.

5.3.4 Effects of TGP on MDA content in corticosterone-treated PC12 cells

As shown in Figure 5.4, the exposure to corticosterone at concentration of 200 μM for 48 h caused a significant increase in the content of MDA (114%), as compared to the control. Co-treatment with TGP (1, 5 and 10 mg/L) significantly decreased MDA content in corticosterone-treated PC12 cells (14%, 25% and 32% respectively), as compared to the corticosterone group.

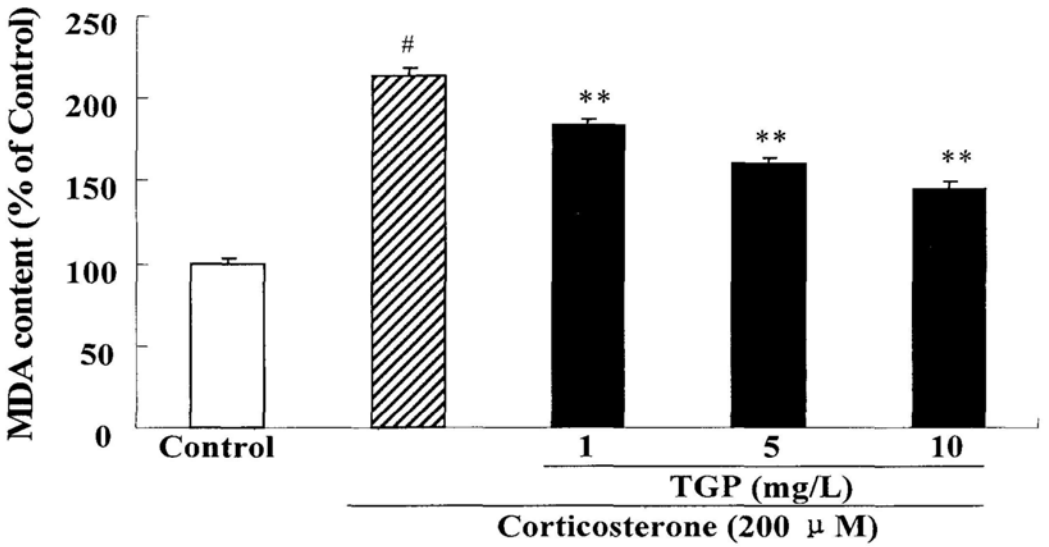


Figure 5.4 Effect of TGP on MDA content in corticosterone-treated PC12 cells.
Values given are the mean ± SEMs (n = 6).
$^{\#}$ p <0.01 as compared to the control; ** p <0.01 as compared to the corticosterone group.

5.3.5 Effects of TGP on GSH content in corticosterone-treated PC12 cells

As shown in Figure 5.5, the exposure to corticosterone at concentration of 200 μM for 48 h caused a significant decrease in the content of GSH (58%), as compared to the control. Co-treatment with TGP (1, 5 and 10 mg/L) significantly increased GSH content in corticosterone-treated PC12 cells (29%, 50% and 68% respectively), as compared to the corticosterone group.

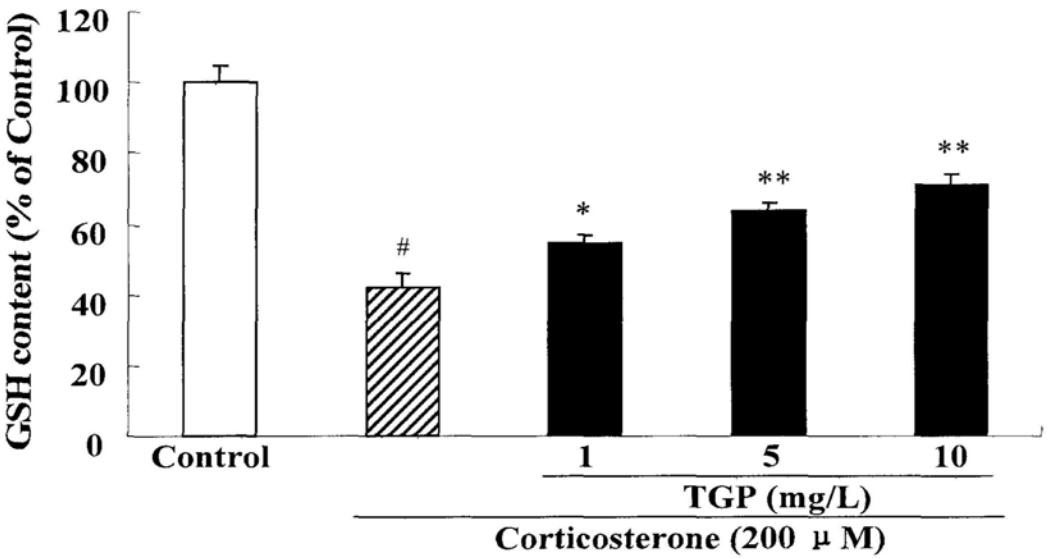


Figure 5.5 Effect of TGP on GSH content in corticosterone-treated PC12 cells. Values given are the mean ± SEMs (n = 6).
$^{\#}p < 0.01$ as compared to the control; $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to the corticosterone group.

5.3.6 Effects of TGP on SOD activity in corticosterone-treated PC12 cells

As shown in Figure 5.6, the exposure to corticosterone at concentration of 200 μM for 48 h caused a significant decrease in the activity of SOD (60%), as compared to the control. Co-treatment with TGP (1, 5 and 10 mg/L) significantly increased SOD activity in corticosterone-treated PC12 cells (32%, 45% and 52% respectively), as compared to the corticosterone group.

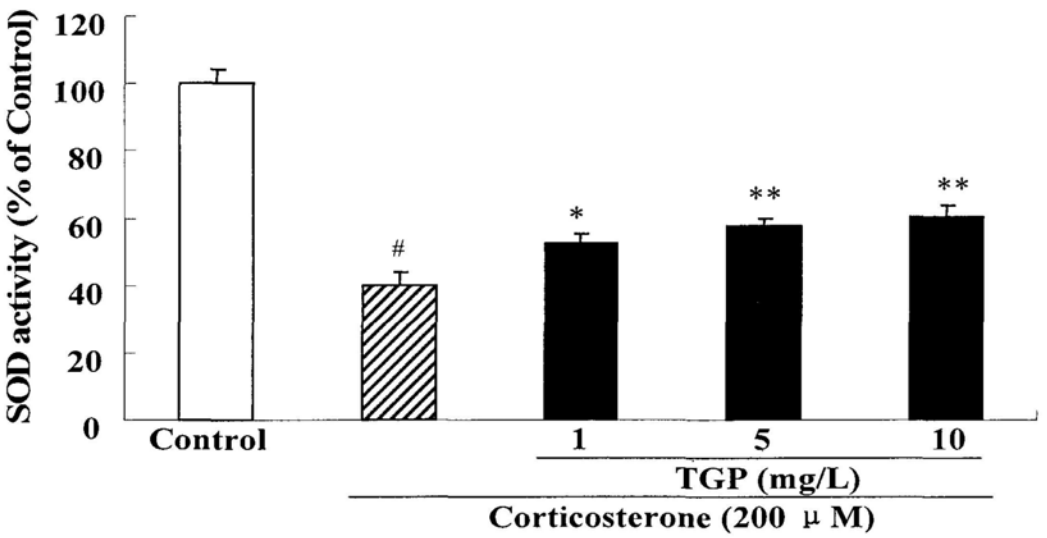


Figure 5.6 Effect of TGP on SOD activity in corticosterone-treated PC12 cells. Values given are the mean ± SEMs (n = 6).
$^{\#}p < 0.01$ as compared to the control; $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to the corticosterone group.

5.3.7 Effects of TGP on CAT activity in corticosterone-treated PC12 cells

As shown in Figure 5.7, the exposure to corticosterone at concentration of 200 μM for 48 h caused a significant decrease in the activity of CAT (57%), as compared to the control. Co-treatment with TGP (1, 5 and 10 mg/L) significantly increased CAT activity in corticosterone-treated PC12 cells (37% and 41% respectively), as compared to the corticosterone group.

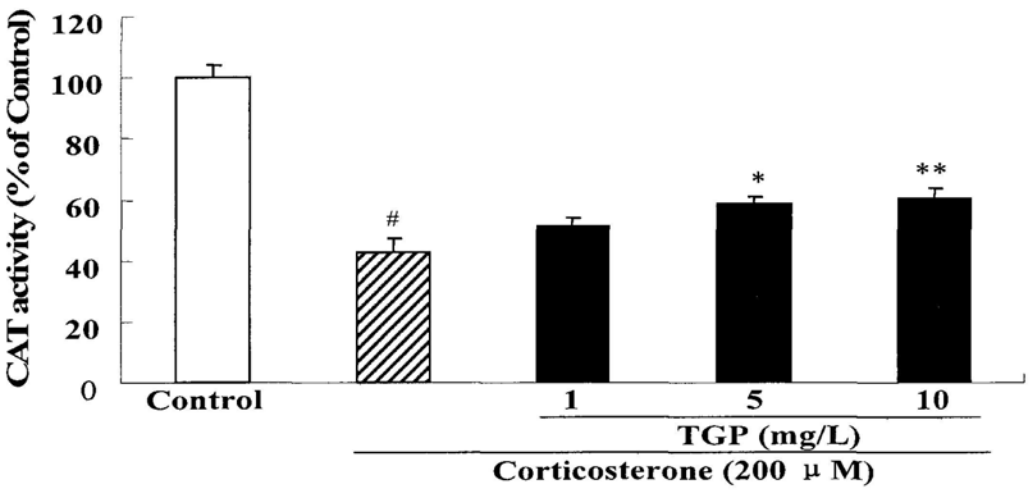


Figure 5.7 Effect of TGP on CAT activity in corticosterone-treated PC12 cells. Values given are the mean ± SEMs (n = 6).
$^{\#}p < 0.01$ as compared to the control; $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to the corticosterone group.

5.3.8 Effect of TGP on BDNF protein and mRNA levels in corticosterone-treated PC12 cells

The protein level of BDNF in PC12 cells when exposed to corticosterone at concentration of 200 μM for 48 h was significantly decreased (35%), as compared to the control (Figure 5.8A). Co-treatment with TGP (1, 5 and 10 mg/L) significantly increased BDNF protein level in corticosterone-treated PC12 cells (25%, 35% and 39% respectively), as compared to the corticosterone group. Similar results were found in BDNF mRNA level (Figure 5.8B). The mRNA level of BDNF in PC12 cells when exposed to corticosterone at concentration of 200 μM for 48 h was significantly decreased (43%), as compared to the control. Co-treatment with TGP (1, 5 and 10 mg/L) significantly increased BDNF mRNA level in corticosterone-treated PC12 cells (23%, 38% and 45% respectively), as compared to the corticosterone group.

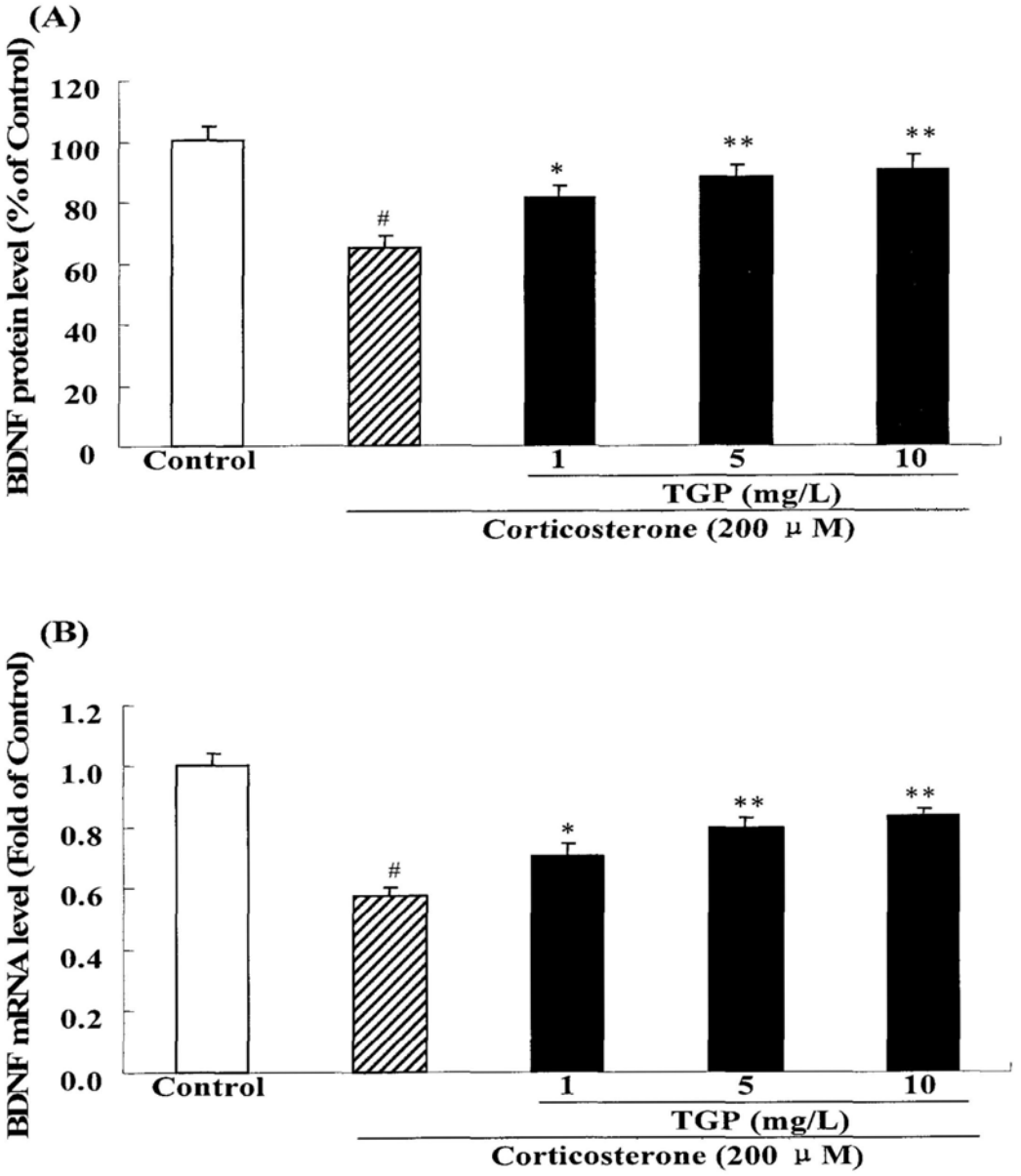


Figure 5.8 Effect of TGP on BDNF protein (A) and mRNA (B) levels in corticosterone-treated PC12 cells.

Values given are the mean ± SEMs (n = 6).

$^{\#}p < 0.01$ as compared to the control; $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to the corticosterone group.

5.3.9 Effect of TGP on NGF protein and mRNA levels in corticosterone-treated PC12 cells

The protein level of NGF in PC12 cells when exposed to corticosterone at concentration of 200 μM for 48 h was significantly decreased (53%), as compared to the control (Figure 5.9A). Co-treatment with TGP (1, 5 and 10 mg/L) significantly increased NGF protein level in corticosterone-treated PC12 cells (25%, 44% and 50% respectively), as compared to the corticosterone group. The mRNA level of NGF in PC12 cells when exposed to corticosterone at concentration of 200 μM for 48 h was significantly decreased (56%), as compared to the control (Figure 5.9B). Co-treatment with TGP (1, 5 and 10 mg/L) significantly increased NGF mRNA level in corticosterone-treated PC12 cells (29%, 59% and 69% respectively), as compared to the corticosterone group.

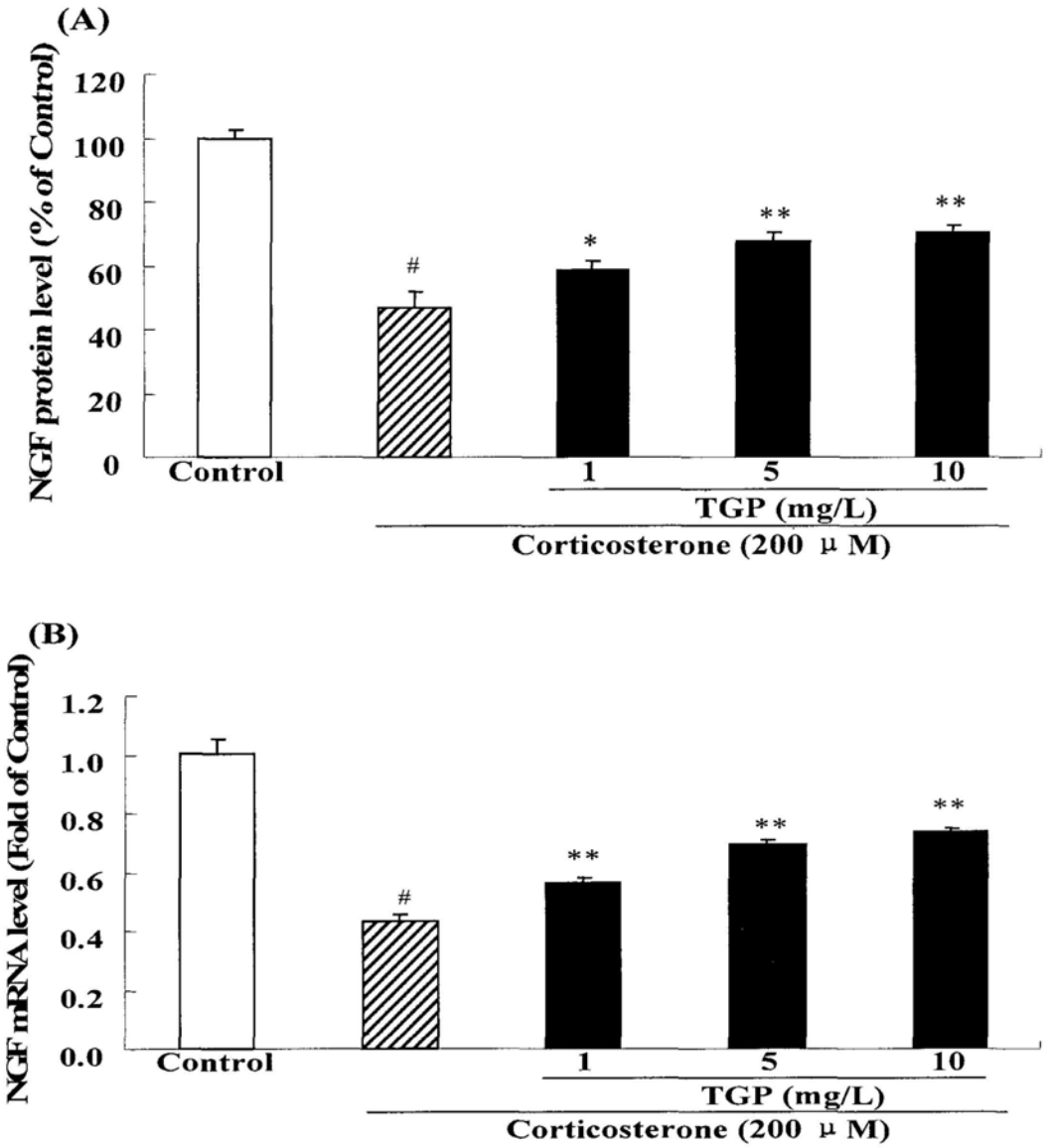


Figure 5.9 Effect of TGP on NGF protein (A) and mRNA (B) levels in corticosterone-treated PC12 cells.

Values given are the mean ± SEMs (n = 6).

$^{\#}$ p <0.01 as compared to the control; $^{*}p$ <0.05, ** p <0.01 as compared to the corticosterone group.

5.3.10 Effect of TGP on NT-3 protein and mRNA levels in corticosterone-treated PC12 cells

The protein level of NT-3 in PC12 cells when exposed to corticosterone at concentration of 200 μM for 48 h was significantly decreased (42%), as compared to the control (Figure 5.10A). Co-treatment with TGP (5 and 10 mg/L) significantly increased NT-3 protein level in corticosterone-treated PC12 cells (42% and 50% respectively), as compared to the corticosterone group. The mRNA level of NT-3 in PC12 cells when exposed to corticosterone at concentration of 200 μM for 48 h was significantly decreased (46%), as compared to the control (Figure 5.10B). Co-treatment with TGP (1, 5 and 10 mg/L) significantly increased NT-3 mRNA level in corticosterone-treated PC12 cells (27%, 45% and 51% respectively), as compared to the corticosterone group.

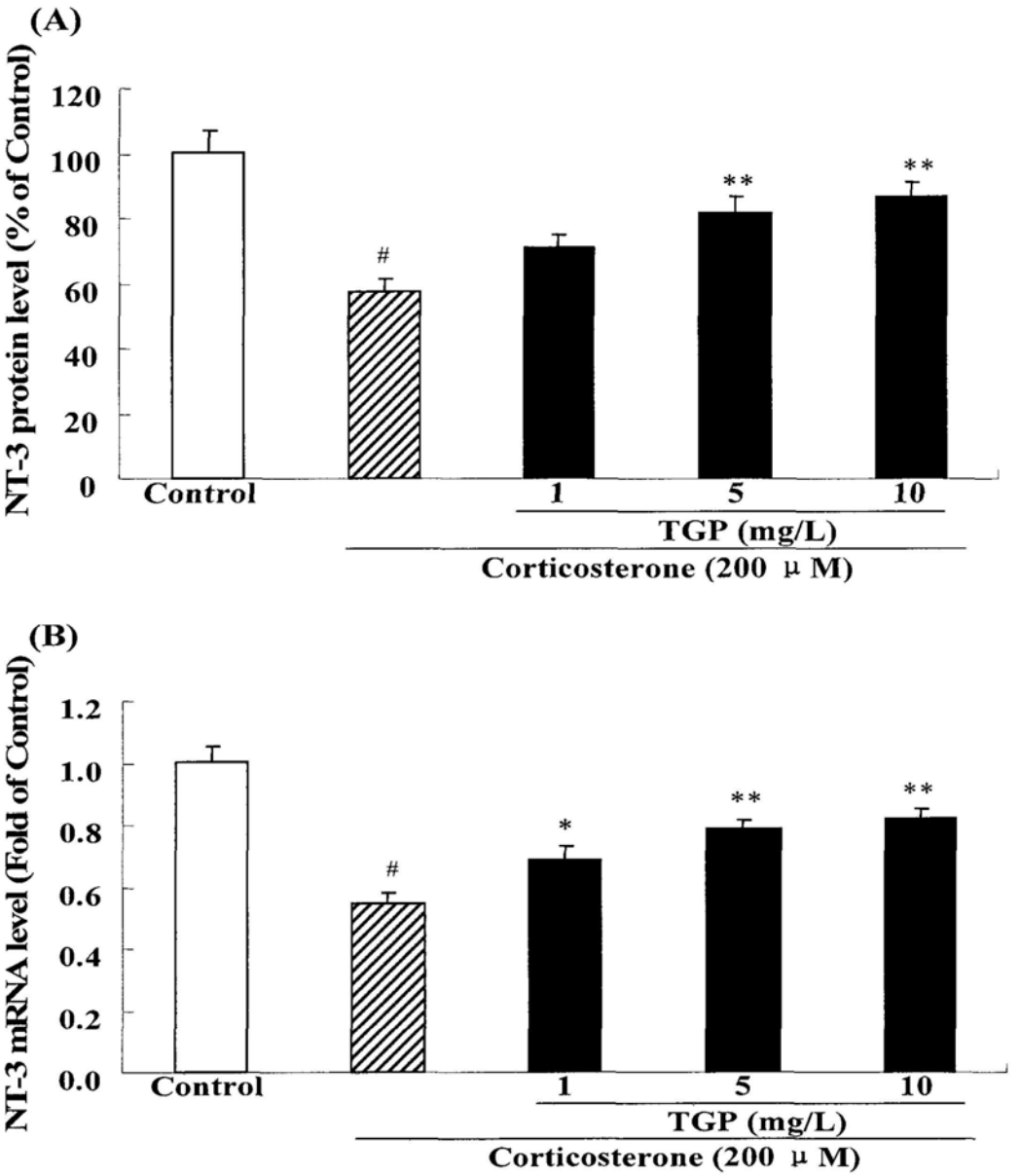


Figure 5.10 Effect of TGP on NT-3 protein (A) and mRNA (B) levels in corticosterone-treated PC12 cells.

Values given are the mean ± SEMs (n = 6).

$^{\#}p < 0.01$ as compared to the control; $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to the corticosterone group.

5.4 Discussion

The hippocampus is one of the brain structures that is important for emotional and cognitive function. Recent clinical studies have demostrated that depression was closely associated with reduced hippocampal volumes, and there was a positive correlation between the hippocampus atrophy and the time course of depression (Li et al., 2003b; MacQueen et al., 2003; Saylam et al., 2006). Postmortem analyses indicated a reduction in the neuronal cells in the hippocampus of patients with depression (Stockmeier et al., 2004). Preclinical studies also showed that the number of hippocampal neuronal cells were significantly decreased in depressive animals (Magariños and McEwen, 1995; Magariños et al., 1999; Zhang et al., 2003; Takuma et al., 2007). High corticosterone level is associated with pathological changes in the hippocampus in experimental depressive animals (Magariños and McEwen, 1995; Sapolsky, 2000; Murray et al., 2008). High concentration of corticosterone has also been shown to induce cytotoxicity in primary cultured hippocampal neurons and PC12 cells, which could be reversed by antidepressants (Gao et al., 2008; Li et al., 2003b and 2003c; Li et al., 2004; Zhu et al., 2006a; Zhu et al., 2006b; Li et al., 2007). Consistent with those reports, the present study demonstrated that treatment of PC12 cells with high concentration of corticosterone (200

μM) for 48 h caused a significant decrease in the cell viability and increase in the LDH leakage, confirming its neurotoxicity in PC12 cells. TGP partly reversed these changes induced by corticosterone, supporting the antidepressant-like effect of TGP *in vivo*.

Oxidative stress, defined as a disturbance in the balance between the production of ROS and antioxidant defenses, has also been showed to be involved in the neuronal injury induced by corticosterone (Goodman et al., 1996; McIntosh et al., 1998a and 1998b; Schmidt et al., 2002; Zafir and Banu, 2009). It has been demonstrated that corticosterone treatment caused a significant increase in the production of ROS (Goodman et al., 1996; Zafir and Banu, 2009). Excessive ROS production can cause damages to major macromolecules in cells, including lipids, proteins, and nucleic acids (Niebrój-Dobosz et al., 2004; Zhao et al., 2008), culminating in neuronal dysfunction and depression. MDA, a by-product of lipid peroxidation, is produced under oxidative stress, reflects oxidative damage of plasma membrane and resultant thiobarbituric acid reactive substances (Xiao et al., 2008). It has been reported that MDA level was increased in corticosterone-treated rat brain (Zafir and Banu, 2009), further providing evidence that corticosterone can induce oxidative stress. The present study also found that treating PC12 cells of corticosterone (200 μM) for 48 h caused a significant increase in the levels of intracellular ROS and

MDA. Co-treatment with TGP significantly decreased in the levels of intracellular ROS and MDA in a concentration-dependent manner. On the other hand, biological antioxidants are natural compounds which can prevent the uncontrolled formation of free radicals and activated oxygen species, or inhibit their reaction with biological structures (Yazdanparast et al., 2008). These compounds include antioxidative enzymes, such as SOD and CAT, and non-enzymatic antioxidants, such as GSH (Yazdanparast et al., 2008). SOD and CAT have been shown to directly catalyze the transformation of peroxides and superoxides to nontoxic species (Griendling and Ushio-Fukai, 2000). GSH, the most abundant natural antioxidant, also play a critical role in the anti-ROS defense system (Mueller et al., 2001). The efficiency of this antioxidant defense system is apparently weakened in depressive disorder (Lukash et al., 2002), resulting in ineffective scavenging of free radicals which may lead to tissue damages (Niebrój-Dobosz et al., 2004; Zhao et al., 2008). Corticosterone treatment also has been shown to cause impairment of antioxidant enzyme defenses and decrease in the content of GSH in rat brain and primary cultured hippocampal neurons (McIntosh et al., 1998a and 1998b; Schmidt et al., 2002; Zafir and Banu, 2009). Consistent with these observations, the present study showed that GSH level, SOD and CAT activities were significantly decreased in 200 μM corticosterone-treated PC12 cells. Moreover, co-treatment with TGP at the doses of 1, 5

and 10 mg/L dose-dependently increased GSH level, SOD and CAT activities in corticosterone-treated PC12 cells.

BDNF, NGF and NT-3 belong to the family of neurotrophins, which modulate neuronal plasticity, inhibit cell death cascades and increase cell survival proteins that are responsible for proliferation and maintenance of central nervous system neurons (Huang et al., 2001; Blum and Konnerth, 2005; Topic et al., 2008; Huston et al., 2009). Interestingly, in recent years, it was also demonstrated that BDNF, NGF and NT-3 are all involved in the pathophysiology of depression (Nestler et al., 2002; Angelucci et al., 2003; von Richthofen et al., 2003; Lang et al., 2004a and b; Dwivedi et al., 2005; Karege et al., 2005b; Duman and Monteggia, 2006; Topic et al., 2008; Huston et al., 2009), and antidepressant treatment might exert their beneficial action by regulating synthesis and/or release of BDNF, NGF or NT-3 (Hellweg 2002; Angelucci et al., 2003; Xu et al., 2003; Zheng et al., 2006; Chen et al., 2007; Li et al., 2007b; Song et al., 2009). Furthermore, it has been shown that corticosterone treatment caused a significant decrease in BDNF mRNA expression in rat brain, while antidepressants treatment significantly increased BDNF mRNA expression in corticosterone-treated rats (Dwivedi et al., 2006). Previous i*n vitro* studies have also demonstrated that corticosterone treatment significantly decrease BDNF, NGF or NT-3

expression in primary cultured hippocampal neurons and PC12 cells, which could be reversed by antidepressants (Li et al., 2003c; Li et al., 2004; Yu et al., 2004; Li et al., 2007b). These data suggested that decreased BDNF, NGF or NT-3 expression may also be involved in the neuronal injury induced by corticosterone. Consistently, in the present study, treatment of PC12 cells with high concentration of corticosterone (200 μM) for 48 h caused a significant decrease in the protein and mRNA levels of BDNF, NGF and NT-3, while treatment with TGP significantly increased BDNF, NGF and NT-3 protein and mRNA levels in corticosterone-treated PC12 cells.

In conclusion, TGP treatment could dose-dependently protect against corticosterone-induced cell death in PC12 cells. The cytoprotection afforded by TGP treatment was associated with the inhibition of oxidative stress, and the up-regulation of BDNF, NGF and NT-3 protein and mRNA levels.

Chapter 6 General Discussion and Future Work

The present work is the first report describing the antidepressant-like effect of total glycosides of peony (TGP) and revealing its action mechanisms by using *in vitro and in vivo* models of depression.

The antidepressant-like effect of TGP was firstly evaluated by using forced swim, tail suspension and open-field tests. Forced swim and tail suspension tests are the widely used animal models of depression for antidepressant drug screening (Porsolt et al., 1977; Steru et al., 1985). Open-field test is designed to measure behavioral responses such as locomotor activity (Herrera-Ruiz et al., 2006). The results showed that intragastric administration of TGP caused a significant reduction of immobility time in both forced swim and tail suspension tests in mice. TGP treatment also significantly reduced the duration of immobility time in the forced swim test in rats. In addition, TGP treatment did not increase locomotor activity in the open-field test in both mice and rats, suggesting that the reduction of immobility time in the forced swim and tail suspension tests was unlikely due to a psychomotor-stimulant effect, but rather an antidepressant-like effect of TGP.

In order to further confirm the antidepressant-like effects of TGP, the antidepressant-like effect of TGP was investigated in a rat model induced by chronic unpredictable mild stress (CUMS). The CUMS-induced depression model is considered as the most promising and valuable rodent model to study depression in animals, mimicking several human depressive symptoms (Willner, 1997 and 2005). The results showed that a 5-week CUMS exposure caused depression-like behavior in rats, as indicated by the significant decreases in sucrose consumption (assessed by sucrose preference test) and locomotor activity (assessed by open-field test), and increase in immobility time (assessed by forced swim test). Intragastric administration of TGP during the five weeks of CUMS significantly suppressed these behavioral changes induced by CUMS, suggesting that TGP produce antidepressant-like effects in the CUMS-induced model in rats.

CUMS-induced depression model has been demonstrated to be suitable for studying the neurobiological basis of depression and the mechanisms of action of antidepressant drugs (Willner, 1997 and 2005). In addition, it has been suggested that neuronal atrophy and destruction in the hippocampus play a causal role in the development and progress of depression and protection against hippocampus damage may be an important therapeutic intervention of depression (Manji and Duman, 2001; Fuchs et al., 2004). Therefore,

whether TGP had neuroprotective effects on the hippocampus of CUMS-treated rats was investigated to throw light on the action mechanisms for the antidepressant-like effects of TGP. The results showed a 5-week CUMS exposure caused a significant decrease in the pyramidal neuronal cells in the hippocampal CA3 region. This damage was associated with the hyperactivation of hypothalamic-pituitary-adrenal (HPA) axis characterized by increased serum corticosterone level and decreased hippocampal glucocorticoid receptor (GR) expression, and increased oxidative stress and decreased neurotrophins such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) expression in the hippocampus. Treatment with TGP significantly increased the pyramidal neuronal cells in the hippocampal CA3 region of CUMS-treated rats, suggesting that TGP produce neuroprotective effects on the hippocampus of CUMS-treated rats. This effect was associated with the attenuation of HPA axis hyperactivation, and the inhibition of oxidative stress and the up-regulation of BDNF and NT-3 expression in the hippocampus.

Finally, the nuroprotective effects of TGP and the possible mechanisms were further investigated in corticosterone-treated rat pheochromocytoma (PC12) cells. Treatment of PC12 cell line with high concentration of corticosterone to simulate the hippocampal nuronal damage in depressive disorder, has been used as an *in vitro* experimental model of

depression. The results showed that treating PC12 cells with corticosterone at concentration of 200 μM for 48 h caused cell death. The cytotoxicity was associated with an increased oxidative stress, and decreased neurotrophins such as BDNF, nerve growth factor (NGF) and NT-3 expression in the cells. TGP treatment protected against the corticosterone-induced toxicity in PC12 cells in a dose-dependent manner. The cytoprotection afforded by TGP treatment was likely associated with an enhancing antioxidant activity, as well as elevation of BDNF, NGF and NT-3 expressions.

In summary, the above findings results have demonstrated an antidepressant-like effects of TGP. The antidepressive action of TGP may be mediated by the modulation of the HPA axis function, the inhibition of oxidative stress, and the up-regulation of neurotrophins, thereby leading to the neuroprotective effects.

Future Work

1. To explore cellular and molecular mechanisms for the antidepressive action of TGP by using Knockout animals;

2. To evaluate the pharmacokinetics of TGP in normal and depressive animals;

3. To evaluate the toxicological profile of TGP.

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