CHAPTER VI
DISCUSSION

Methamphetamine (METH) is an illicit CNS stimulant commonly abused in Thailand and worldwide. After a long-term use, METH induces neurotoxicity causing permanent loss of neurons in the central dopaminergic pathway. The neurotoxic effects of METH have been associated with the formation of toxic reactive oxygen species (ROS) and subsequent activation of apoptosis (Wu, Ping et al. 2007). The form of METH tables that is circulated in the market, so called Ya-ba, contains only 20-25% of METH by weight. The rest of the mass contains caffeine (CAF), a xanthine stimulant drug, in order to increase the bulk of the formulation as well as to enhance CNS stimulating effects (Puthaviriyakorn, Siriviriyasomboon et al. 2002). The recent study from our lab demonstrated that combined treatment of METH and caffeine at non-toxic concentration of each chemical significantly increases the toxic effects both *in vivo* and *in vitro* (Sinchai, Plasen et al. 2011). This study demonstrated that caffeine probably potentiates the toxic effects of methamphetamine by increasing in the release of dopamine and the excess in ROS generation. However, the underlying mechanism is not clearly understood. It has been known that both autophagy and apoptosis are important in maintain cellular homeostasis and both can be triggering by ROS. Therefore we aimed to investigate whether the potentiating effect of caffeine is enhanced through the modulation of autophagy and apoptosis pathways.

First experiment, we studied the neurotoxic effect of METH in human neuroblastoma, SH-SY5Y culture cells that expresses dopaminergic activity. METH treatment for 24 h decreased cell viability in a dose dependent manner. In the experiment, METH at 0.5 mM which was not alter cell viability and METH at 2 mM which significantly decreased viability to 60% were used as the non toxic dose and the toxic dose of METH, respectively. The concentration of METH and exposure time to cause toxicity are variable, it depends on the types or species of the cells or animals.

To confirm the potentiating effect of caffeine on METH induced neurotoxicity, we examined the cell viability of cell with combined treatment of METH and caffeine. Caffeine at 1 mM which was not alter cell viability and at dose 5 mM which decreased viability of cells to 60% were used as the non toxic dose and the toxic dose of caffeine, respectively. Combination treatment of METH and caffeine significantly increased the toxicity to cells compared the METH treatment alone. Moreover, combined treatment of METH and caffeine at individual non-toxic dose (0.5 mM METH and 1 mM caffeine) can produced the toxic effect to cells. This result demonstrated the potentiating effect of caffeine on METH-induced neurotoxicity confirming the pervious result from our lab which performed in SK-N-SH cells, even the dose of METH used in the experiment might vary from the difference of cells types (Sinchai, Plasen et al. 2011).

The involvement of autophygy and apoptosis pathways on the neurotoxic effect of METH was studied using western blot analysis. To study the involvement of autophagy, we observed the expression of LC3II, p-mTOR, and p-4Ebp1. LC3 (microtubule-associated protein 1 light chain 3, a mammalian homologue of yeast Atg8) is the widely used marker to study autophagy, as it is the only known protein that specifically associates with autophagosomes and not with other vesicular structures. LC3II (16 KDa) which is the form that inserted to autophagosome was used for marker of autophagosome. We observed mammalian target of rapamycin (mTOR), a protein kinase that is a master negative regulator of autophagy and p-4Epb1, a downstream substrate of mTOR which mirrors the mTOR kinase activity for determining autophay induction process. For study the involvement of apoptosis pathway, we observed the expression of cleaved caspase-3 (17/19 kDa), which possesses the protease activities that are important to execute apoptosis. It has been found in this study that METH increased the accumulation of LC3II in SH-SY5Y cells with the peak can be seen at 6 h after the treatment. Exposure to METH at both toxic and non-toxic dose for 6 h cause induction of autophagy and accumulation of autophagosome, as shown in the reduction of p-mTOR and p-4Ebp1 expressions and the increase in LC3II levels, respectively. METH exposure was also induced apoptosis in cells as presented by the increase in the activation of caspase-3.

To determine the role of autophagy on METH-induced neurotoxicity, whether it plays detrimental or protective role on dopaminergic neuronal cell line, we inhibited the autophagy process and observed the viability of cells. Pretreatment of 3-MA, the PI3k inhibitor, was used to inhibit autophagy in our experiment. It has been reported that induction of autophagy is positively regulated by phosphatidylinositol-3-phosphate kinase (PI3k) class III (Vps34) and is negatively regulated by PI3k class I (Castino, Fiorentino et al. 2011). The PI3k inhibitor 3MA can inhibit autophagy, if used under appropriate concentration and time of incubation that preferentially inhibit only PI3k class III (Wu, Tan et al. 2010). In the condition that autophagy was inhibited, as confirm by the lowering of LC3II protein levels, we found the significant reduction of cell viability in METH treated cells associated with the increasing of caspase-3 activation. This result suggested that autophagy play protective role on METH induced neurotocixity and the toxic effect of METH was due mainly to induction of apoptosis. These are in line with the study of Isidoro et al. (2008) which proposed that autophagy exerts a protective mechanism to remove protein aggregates and (bax-positive) mitochondria, which preventing onset of apoptotic cell death in METH-induced neurotoxicity (Castino, Lazzeri et al. 2008).

Next, we studied the involvement of autophygy and apoptosis pathways on the potentiating effect of caffeine on METH-induced neurotoxicity using western blot analysis. Both toxic and non-toxic dose of METH and caffeine showed the same pattern of effect, but slightly difference in the degree of response. Combination treatment of caffeine with METH results in reduction of autophagosome level, as shown in the lowering of LC3II protein level, but the induction of autophagy was increased, as shown by the lowering of p-mTOR and p-4Epb1 levels compared to METH treatment alone. The two results seemed to be contradicted. However, it has to be noted that the number of cellular autophagosome is not represent autophagic flux which is a dynamic process of autophagy begin with autophagosome synthesis, delivery of autophagic substrates to the lysosome, and degradation of autophagic substrates inside the lysosome indicating the cellular autophagic activity. Autophagosome is an intermediate structure in a dynamic pathway, the number of autophagosomes observed at any specific time point is a function of the balance between the rate of their generation and the rate of their conversion into

autolysosomes (Mizushima, Yoshimori et al. 2010). Thus, lowering of autophagosome level can affect from both inhibition of autophagy and also the increasing of autophagosome degradation. To determine whether caffeine inhibited autophagy or interfered with the degradation process, the cells were exposed to combined treatment of METH and caffeine in the presence of ammonium chloride. This drug impairs the activity of acid hydrolases and the fusion of autophagosomes with lysosomes by raising the internal vacuolar pH, thus preserving LC3 II from lysosomal degradation (Castino, Fiorentino et al. 2011). In this condition, autophagosome degradation was inhibited, it has been found that caffeine did not lower the level of LC3II protein in METH treated cells. Thus, caffeine did not inhibit autophagy but may increase the autophagosome degradation rate. All together from western blot analysis of LC3II, p-mTOR, and p-4Ebp1, we provide the information that caffeine increases autophagic flux on METH treated cells. It both increased induction of autophagy and increased autophagosome degradation process.

In the morphology observation, METH at toxic dose induced massive cytoplasmic vacuolisation with in cell while combined treatment of caffeine lowing number of this vacuole. It is possible that this cytoplasmic vacuole might have some relations to autophagosome accumulating in cell, as autophagosome is decreased when treatment with caffeine. However, recent studies stated that these cytoplasmic vacuole is not an autophagosome as it did not colocalized with LC3II, instead it is positive to lysosome membrance marker (LAMP2), indicating the lysosomal nature of these vacuole (Nara, Aki et al. 2012; Funakoshi-Hirose, Aki et al. 2013).

The role of autophagy on caffeine enhanced METH-induced neurotocixity was studied by observed cell viability in cells pretreatment with 3-MA, an autophagy inhibitor. In condition that autophagy was inhibited, we found significant reduction of cell viability in combined treatment of METH and caffeine. This result suggested that the increasing autophagy flux effect of caffeine play protective role on the toxic effect of METH and caffeine combination.

In addition to the increasing of autophagic flux effect, caffeine also increased the induction of apoptosis, as demonstrated by the increase in cleaved caspase-3 expression, revealed the cause of potentiating effect of caffeine on METH toxicity. This result is consistent with the study from Sinchai et al. (2011), which

demonstrated the increase in Bax/Bcl2 ratio in SK-N-SH cells after combined treatment of METH and caffeine (Sinchai, Plasen et al. 2011). It is likely that the toxic effects of the combination are mediated via the oxidative stress and apoptotic pathways. The toxic effect of METH in dopaminergic cell was believed to cause by METH triggers an excessive release of DA which rapidly oxidized by either enzyme or auto-oxidation to form ROS such as superoxide radicals, hydroxyl radicals, hydrogen peroxide, and dopamine quinones which all are toxic to neurons. The enhancement by CAF of METH-induced toxicity was thought to mediated through the action of caffeine as a nonselective antagonist of adenosine A1/A2A receptors. It was reported that systemic administration of caffeine produces a significant increase in the extracellular concentration of dopamine in the striatum. It is possible that the free radicals generated from the increase in dopamine from combination treatment of METH and caffeine may damage neurons by inducing oxidative stress, thus triggering apoptosis cell death (Borycz, Pereira et al. 2007).

The Induction of apoptosis in combined treatment of caffeine with METH together with the increasing of caspase-3 level when autophagy was inhibited in cell exposure to combined treatment of METH and caffeine confirmed that the increase in autophagic flux effect of caffeine help protect cell from apoptosis cell death. Autophagy is an intracellular degradation process which is vital important in maintaining cellular homeostasis. Autophagy antagonizes apoptotic cell death by promoting cell survival, through, for example, the removal of damaged organelles those are a source of genotoxic ROS, or by catabolizing cellular macromolecules to provide a source of nutrients and energy for the starved cell, or by limiting cell stress through the degradation of unfolded protein aggregates. These functions block the stimuli that would trigger an apoptotic response (Eisenberg-Lerner, Bialik et al. 2009). The relationship between autophagy and apoptosis is quite complicated and they share many of the same molecular regulators. Many studies proposed that autophagy acts as an antagonist to block apoptotic cell death by promoting cell survival like what we found in our study, on the other hand, many studies proposed that apoptosis and autophagy can act as partners to induce cell death in a coordinated manners (Nakaso, Ito et al. 2008; Castino, Bellio et al. 2010; Saiki, Sasazawa et al. 2011). However, the

cell decides which pattern to use, depending on the nature of the stimulus and in particular the cell environment (Eisenberg-Lerner, Bialik et al. 2009).

In conclusion, the results of present study provide insight into the effect of caffeine, which was main additive of Ya-Ba, on METH-induced neurotoxicity. Our study proposed the dual effects of caffeine on this toxicity. First, caffeine causes the increase in induction of apoptosis pathways that can potentiate METH-induced neurotoxicity. Second, caffeine can also increase autophagic flux which plays protective role against toxicity caused by its combination with METH, however, the increasing autophagy flux may not be enough to circumvent the stimuli triggering an apoptotic response, since the cell death still be observed. It is interesting that increase induction of autophagy would be the solution for attenuating the toxicity of METH. In additon, it has to be noted that the optimum of various conditions is important for study the roles of autophagy in cell death or survival.