# CHAPTER V
# RESULTS

## 5.1 The cytotoxicity of METH

To determine the cytotoxic effect of METH on SH-SY5Y, cells were incubated with 0 - 4 mM of METH for 24 h before measuring the cell viability by MTT assay. The result was represented as percentage of untreated control. METH caused toxicity to the SH-SY5Y cells in a dose-dependent manner (figure 5.1). The viability of the cells was decreased to 88.66 ± 3.59, 63.40 ± 2.27, 42.47 ± 1.06, and 30.55 ± 1.91 % of control following treatment with 1, 2, 3 and 4 mM of METH, respectively. METH at 0.5 mM was not toxic to cells, while METH at 1 to 4 mM were significantly decreased the cell viability compared with the untreated control. Based on these results, METH at 0.5 mM was used in the subsequent experiments as non-toxic dose of METH and METH at 2 mM, which reduced the viability to 60% of the control, was used as toxic dose of METH.

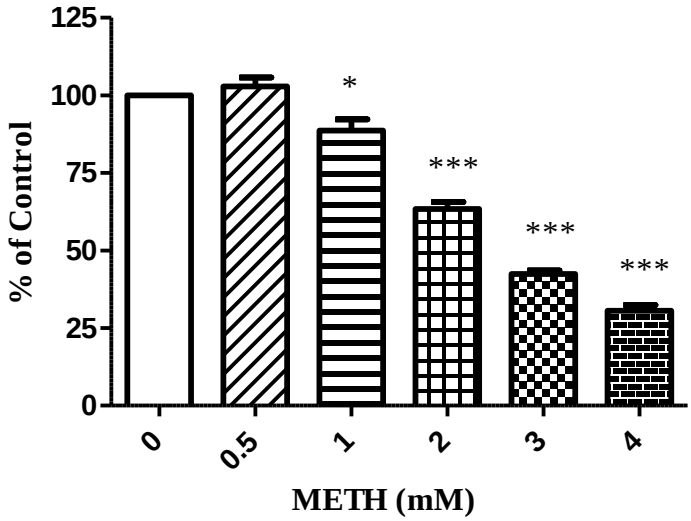



Figure 5.1 The effect of METH on cell viability. SH-SY5Y cells were treated with various concentrations of METH for 24 h. Cell viability was assessed using MTT assay and presented as percentage of untreated (0 mM METH) control. The results were expressed as mean ± SEM of at least three independent experiments. The ANOVA was performed for statistical analysis ($^{*}p < 0.05$ and $^{***}p < 0.001$ compared with control).

## 5.2 The cytotoxicity of caffeine

To determine the cytotoxic effect of caffeine on SH-SY5Y, the cells were incubated with 0 - 20 mM of caffeine for 24 h before measuring the cell viability by MTT assay. The result was represented as percentage of untreated control. Caffeine at the concentration of 0.5 and 1 mM did not alter the cell viability but at 2.5, 5, 10, and 20 mM, the cell viability was decreased to 72.82 ± 2.56, 65.68 ± 1.41, 46.67 ± 2.28, and 35.69 ± 4.08 % of control, respectively (figure 5.2). Based on these results, caffeine at the concentration of 1 mM was used in the subsequent experiments as non-toxic dose of caffeine and caffeine at the concentration of 5 mM, which reduced the viability to 60% of the control, was used as toxic dose of caffeine.

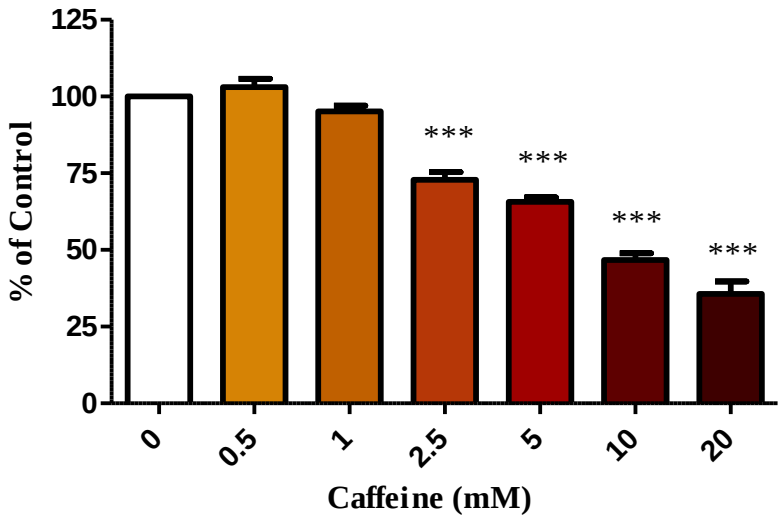



Figure 5.2 The effect of caffeine on cell viability. SH-SY5Y cells were treated with various concentrations of caffeine for 24 h. Cell viability was assessed using MTT assay and presented as percentage of untreated (0 mM caffeine) control. The results were expressed as mean ± SEM of at least three independent experiments. The ANOVA was performed for statistical analysis ($^{***}p < 0.001$ compared with control).

## 5.3 Potentiating effect of caffeine on METH-induced neurotoxicity.

To determine whether caffeine potentiates the METH-induced neurotoxicity, SH-SY5Y cells were treated with METH at the concentration of 0.5 and 2 mM combined with caffeine at the concentration of 1 and 5 mM for 24 h. After incubation, the cell viability was determined using MTT assay. Cell viability was decreased to 86.57 ± 2.89 and 53.38 ± 1.79 % of control in the treatment of 0.5 mM METH in combination with 1 and 5 mM of caffeine and cell viability was decreased to 45.73 ± 1.08 and 33.07 ± 4.04 % of control in the treatment of 2 mM METH in combination with 1 and 5 mM of caffeine, respectively (Figure 5.3). Both toxic and non-toxic dose of METH and caffeine showed the same pattern of effect, but slightly difference in the degree of response. Result of combined treatment demonstrated that the caffeine increased the toxicity of METH when compared to the treatment of METH without caffeine. Moreover, the combined treatment of METH and caffeine at individual non-toxic dose (0.5 mM METH and 1 mM caffeine) become toxic to cells.

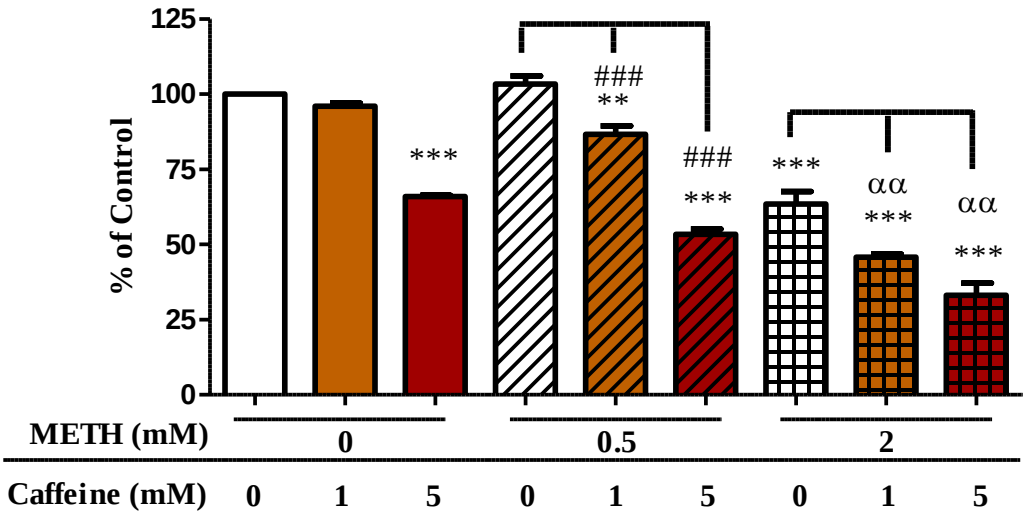



Figure 5.3 Effect of METH and caffeine co-treatment on the viability of SH-SY5Y cells. SH-SY5Y cells were co-treated with METH 0.5 and 2 mM, and caffeine at 1 and 5 mM for 24 h. Cell viability was assessed using the MTT assay and presented as percentage of untreated control group. The results were expressed as mean ± SEM of at least three independent experiments. The ANOVA was performed for statistical analysis ($^{**}p < 0.01$ and, $^{***}p < 0.001$ compared with untreated control, $^{###}p < 0.001$ compared with sample treated with METH 0.5 mM, $^{αα}p < 0.01$ compared with sample treated with METH 2 mM).

## 5.4 The neurotoxic effect of METH on autophagy pathway

### 5.4.1 Effect of METH on LC3-II expression

In this study, we determined the neurotoxic effect of METH on autophagy pathway in SH-SY5Y cells using western blot analysis. The level of the microtubule-associated protein light chain 3 (LC3-II), an autophagosome protein was served as marker of autophagy. SH-SY5Y cells were grown on 6 well plates at density of $9\times10^5$ cells/well for 24 h prior to treatment with METH 2 mM. Protein samples were collected at 0.5, 1, 3, and 6 h. The expression of LC3II after METH treatment was shown in figure 5.4. The level of LC3II was increased with time and was peaked at 6 h. This time point was used in the subsequent experiments.

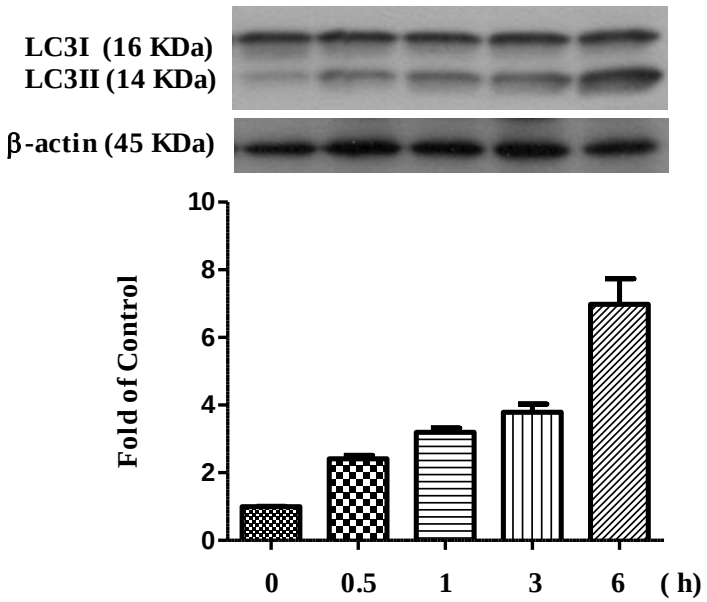



Figure 5.4 The effect of METH on LC3 expression. SH-SY5Y was treated with 2 mM of METH for a period of time. Samples were collected at indicated time point. The LC3-II protein levels were measured by western blot analysis. β-actin was used as a loading control.

### 5.4.2 Role of autophagy on the toxic effect of METH

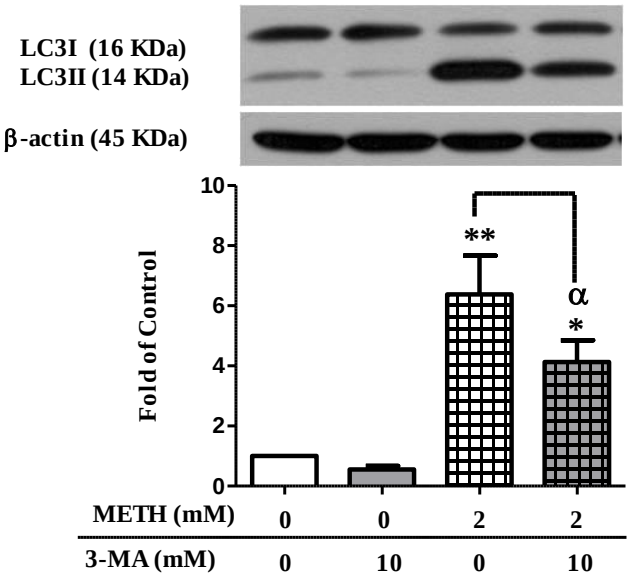
To determine whether autophagy plays detrimental or protective role on the toxic effect of METH on dopaminergic neuronal cell line, SH-SY5Y cells were pretreatment with an inhibitor of class III phosphoinositide 3-kinase (PI3K) and an autophagy inhibitor, 3-Methyladenine (3-MA), for 1 h before exposure to METH.

5.4.2.1 Effect of 3-MA on LC3II and caspase-3 expression in METH treated SH-SY5Y cells.

Effect of 3-MA on LC3II and caspase-3 expression in METH treated SH-SY5Y cells was determined by western blot analysis. After incubating the cells with 2 mM METH for 6 h, the level of the LC3-II, was increased compare to untreated cells. METH-induced increases in LC3-II levels were blocked by addition of 10 mM 3-MA, 1 h prior to treat cells with METH 2 mM for 6 h (Figure 5.5).

The amount of cleaved caspase-3 protein levels in METH-induced toxicity in SH-SY5Y cells was determined using western blot analysis. The results showed that blocking autophagy in SH-SY5Y cells with 3-MA increased the amount of cleaved caspase-3 when compared to cells only treated with METH (Figure 5.5).

A)

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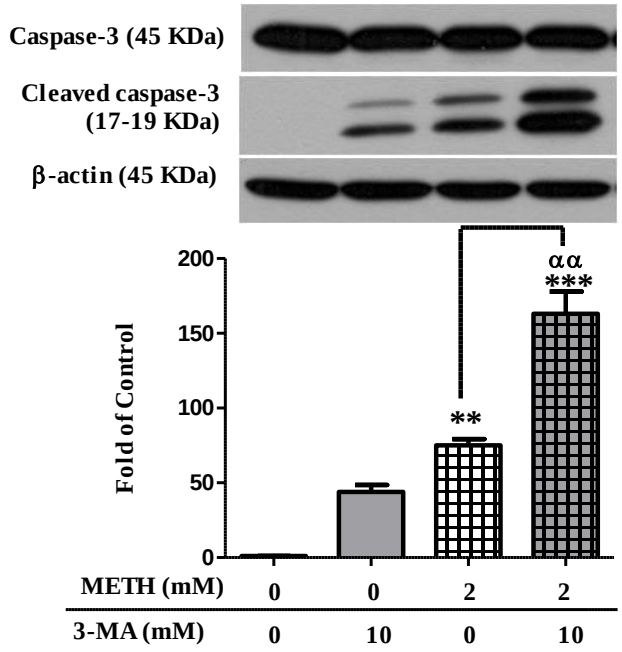


Figure 5.5 Effect of METH-induced increase in LC3II protein levels and effect of 3-MA-induced induction of cleaved caspase-3 protein levels in SH-SY5Y cultured cells. Cells were treated with 2 mM METH for 6 h with or without pretreatment with 10 mM 3-MA for 1 h. The amount of LC3II (A) and cleaved caspase-3 (B) were determined using Western blot analysis. β-actin was used as a loading control. The ANOVA was performed for statistical analysis ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with untreated control, $^{\alpha}p < 0.05$, and $^{\alpha\alpha}p < 0.01$ compared with sample treated with METH 2 mM).

5.4.2.2 Effect of 3-MA on cell viability in METH treated SH-SY5Y cultured cells

SH-SY5Y cells were exposed to 0.5 and 2 mM of METH for 24 h with or without pretreatment with 3-MA at 5 and 10 mM for 1 h. After incubation, the cell viability was determined using MTT assay. Since 3-MA itself slightly affected cell viability, in order to clearly observed the autophagy inhibition effect to cell viability, the toxicity of METH in the presence of 3-MA was compared to the viability of cells treated with 3-MA alone. METH at 0.5 mM was not toxic to cells (102.30 ± 3.17%) when compared with the untreated control. Pretreatment with 5 and 10 mM of 3-MA prior to 0.5 mM of METH caused reduction of cell viability to 95.81± 3.27% and 86.39 ± 3.12% of the control, respectively. METH at 2 mM decreased cell viability to 61.76 ± 1.38% of the control. 3-MA enhanced the cytotoxic effect of METH 2 mM, by which, 5 and 10 mM of 3-MA decreased the cell viability to 59.13 ± 1.16% and 37.51 ± 2.59% of the control values, respectively (Figure 5.6B). The results showed that inhibition of autophagy by pretreatment with 3-MA in METH-treated cells decreased cell viability compared to METH treatment alone. These suggested that autophagy play protective role on the toxic effect of METH on dopaminergic neuronal cell lines, SH-SY5Y cells.

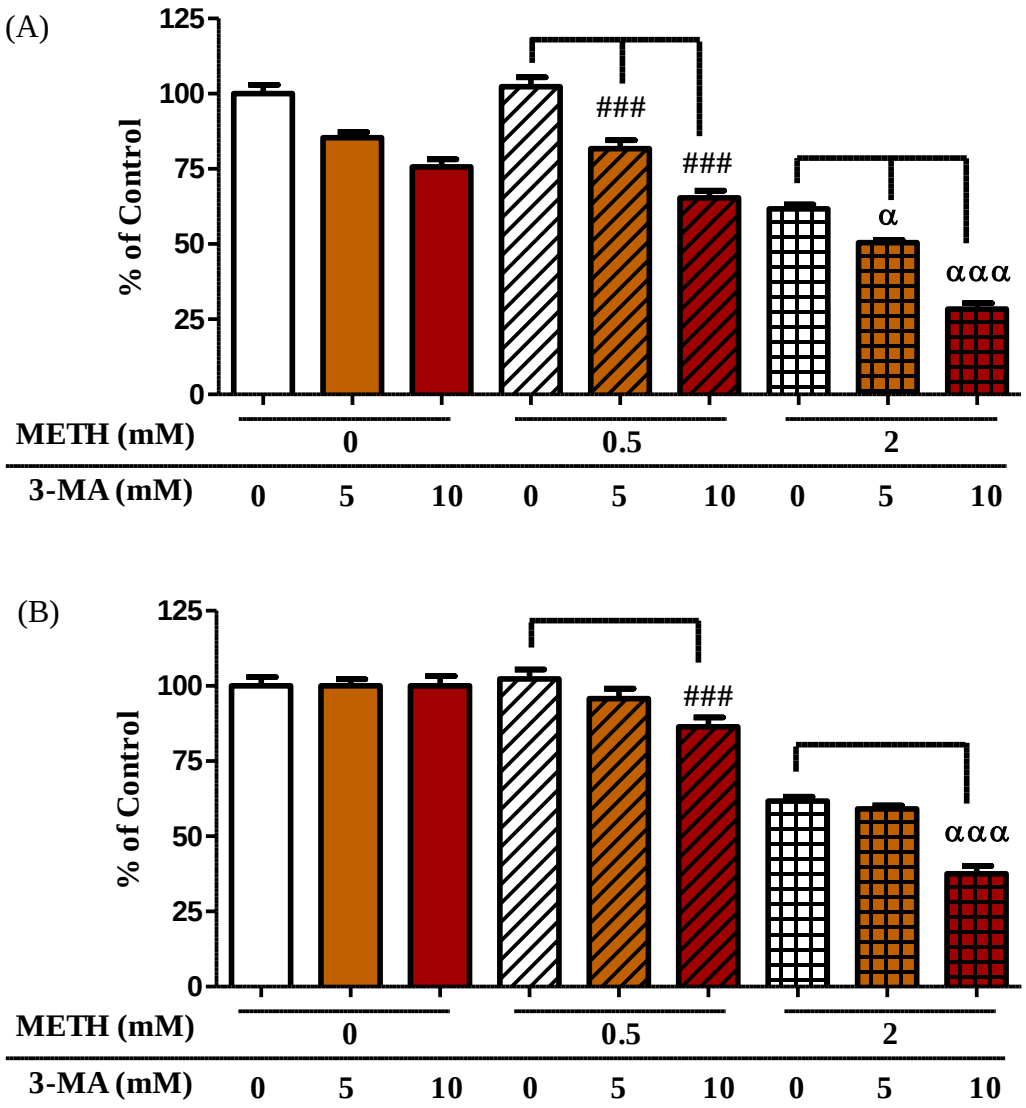


Figure 5.6 Effect of autophagy inhibitor, 3-MA, on cell viability in SH-SY5Y cultured cells. SH-SY5Y cells were treated with 0.5 and 2 mM of METH for 24 h with or without pretreatment with 5 and 10 mM of 3-MA for 1 h. Cell viability was assessed using the MTT assay. (A) The results were presented as percentage of untreated control. (B) After adjusting the percentage in the histogram, the toxicity of METH in the presence of 3-MA were expressed as percentage of cells viability in 3-MA treatment alone. The ANOVA was performed for statistical analysis ( $^{###}p <$ 0.001 compared with sample treated with METH 0.5 mM, $^{ααα}p < 0.001$ compared with sample treated with METH 2 mM).

## 5.5 The involvement of autophagy and apoptosis pathways on potentiating effect of caffeine in METH-induced neurotoxicity

### 5.5.1 Effect of METH on LC3-II, p-mTOR, p-4Ebp1, claved caspase-3 expression

In this study, we determined whether caffeine enhanced METH-induced neurotoxicity through modulation of autophagy pathway. The expression of LC3II protein which represents autophagosome level, the expression of p-mTOR which is a master negative regulator of autophagy and the expression of p-4Ebp1 which is downstream substrate of mTOR were determined using western blot analysis. The involvement of apoptosis pathway on this potentiating effect was also determined by observing the expression of cleaved caspase-3, which possesses the protease activities that are important to execute apoptosis.

5.5.1.1 LC3II expression

After incubating the cells with METH (0.5 and 2 mM) and caffeine (1 and 5 mM) for 6 h, the level of LC3-II was increased in dose dependent manner compare to untreated control. Combined treatment of METH and caffeine showed reduction in LC3II protein level compare to cells treated only with METH, the reduction in LC3II levels was marked at high dose of METH (2mM) treatment (Figure 5.7). This result showed that autopohagosome was increased in cells treated with METH compared to untreated control but this autophagosome was decreased when combined treatment the cells with caffeine although caffeine itself increased autophagysome compared to untreated control.

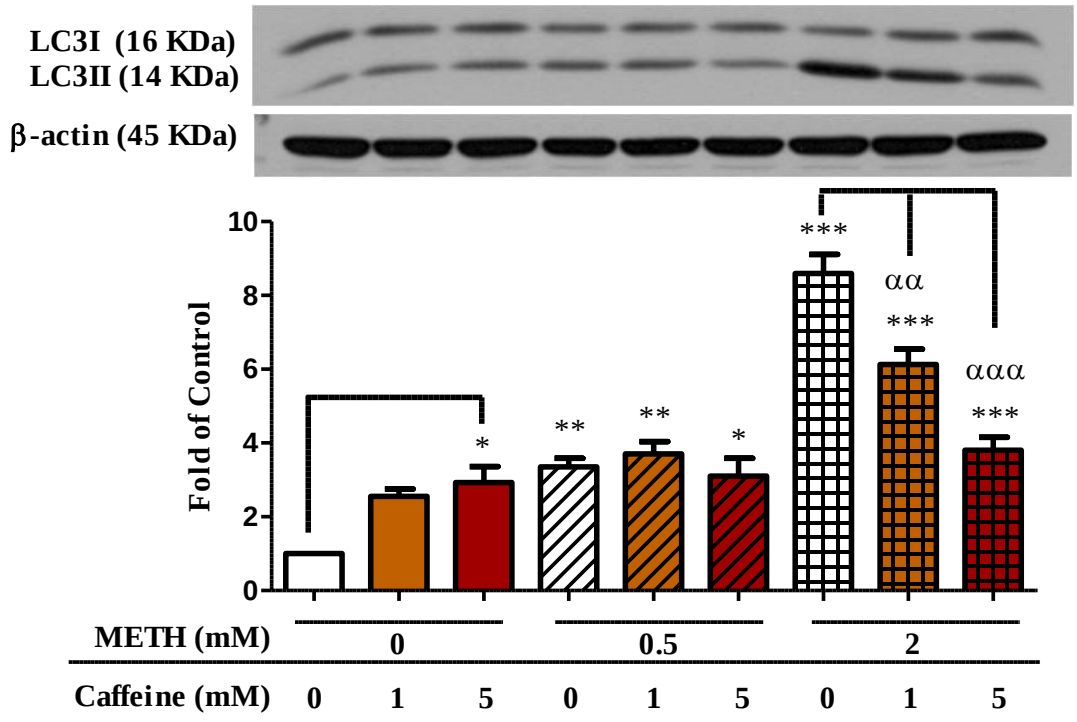


Figure 5.7 Effect of co-treatment with METH and caffeine on LC3II protein levels in SH-SY5Y cultured cells. Cells were treated with various conditions of co-treatment for 6 h. The amount of LC3II was determined using Western blot analysis. β-actin was used as a loading control. The ANOVA was performed for statistical analysis ($^{*}p< 0.05$, $^{**}p < 0.01$, and $^{***}p <0.001$ compared with control, $^{\alpha\alpha}p < 0.01$, and $^{\alpha\alpha\alpha}p < 0.001$ compared with sample treated with METH 2 mM).

5.5.1.2 p-mTOR and p-4Epb1 expression

Cells were incubated with METH (0.5 and 2 mM), caffeine (1 and 5 mM), and combined treatment of METH and caffeine for 6 h. Both toxic dose of METH and caffeine significantly decreased the level of p-4Ebp1 protein. The decrease in the level of p-4Ebp1 was more obvious after the cells were treated with combined treatment of METH and caffeine. P-mTOR expression analysis produced the same pattern of effect with p-4Epb1 expression, however, not statistical significant (figure 5.8). These results demonstrated that autophagy can be induced by both METH and caffeine and the induction of autophagy was increased in combined treatment of METH and caffeine.

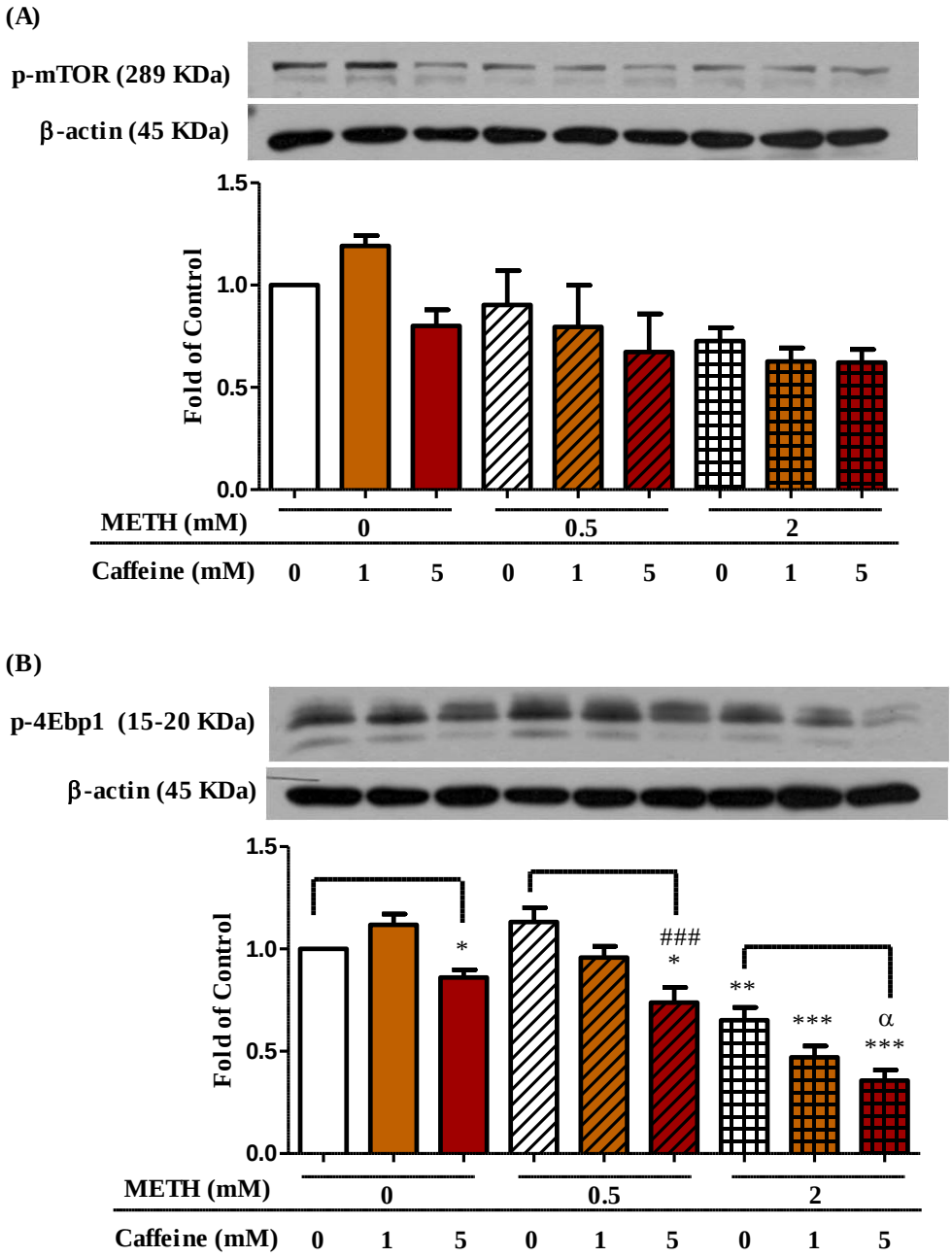


Figure 5.8 Effect of co-treatment between METH and caffeine on p-mToR and p-4Ebp1 protein levels in SH-SY5Y cultured cells. Cells were treated with various condition of co-treatment for 6 h. The amount of p-mToR (A) and p-4Ebp1 (B) was determined using Western blot analysis. β-actin was used as a loading control. The ANOVA was performed for statistical analysis ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with control, $^{\#\#\#}p < 0.001$ compared with sample treated with METH 0.5 mM, $^{\alpha}p < 0.05$ compared with sample treated with METH 2 mM).

5.5.1.3 Effect of co-treatment between METH and caffeine in the presence of ammonium chloride on the levels of LC3II protein.

Caffeine caused reduction of LC3II expression from western blot analysis, which suggested toward autophagy inhibition, while the result of mTOR and 4Ebp1 expressions suggested toward autophagy induction. Therefore, we performed the other experiment to see whether the reduction of autophagosome in combination treatment of METH and caffeine from LC3 expression analysis is due to the inhibition of autophagy or the increase in autophagosome degradation process. Cells were pretreated with 10 mM ammonium chloride ($NH_4Cl$) for 1 h prior to treated with METH and caffeine. $NH_4Cl$ 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 the presence of $NH_4Cl$, which inhibit autophagosome degradation process, combined treatment of caffeine on METH treatment did not alter LC3 protein level compared to the treatment of METH alone (figure 5.9). Thus, the lowering of LC3 protein level when treated the cells with caffeine and METH treatment (without $NH_4Cl$) did not due to inhibition of autophagy induction but may come from the increase in autophagosome degradation process caused by caffeine.

In summary, the analysis of protein involving in autophagy by determining the band of LC3II, mTOR, and 4Ebp1 expression suggested that caffeine increased autophagy flux. Caffeine both induced autophagy and increase autophagosome degradation in METH treated cells.

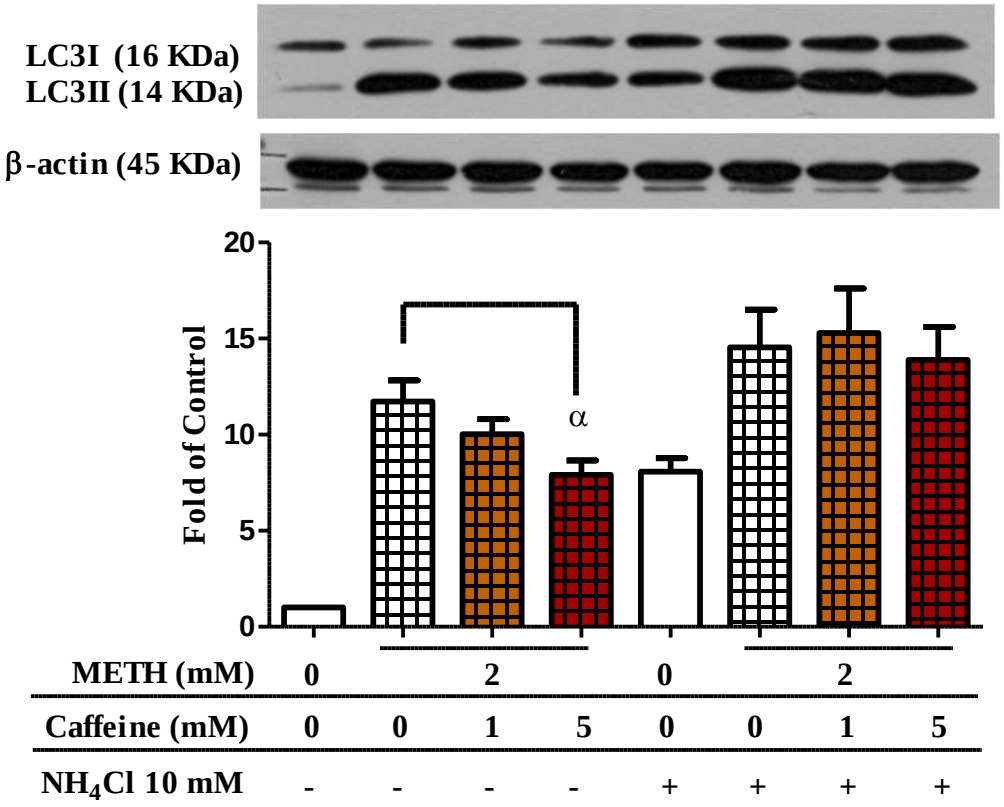


Figure 5.9 Effect of co-treatment between METH and caffeine in the presence of ammonium chloride on LC3II protein levels in SH-SY5Y cultured cells. Cells were treated with various conditions of co-treatment for 6 h. The amount of LC3II was determined using Western blot analysis. β-actin was used as a loading control. The ANOVA was performed for statistical analysis ($^{\alpha}p< 0.05$ compared with sample treated with METH 2 mM).

5.5.1.4 Cleaved caspase-3 expression

After incubating the cells with METH (0.5 and 2 mM) and caffeine (1 and 5 mM) for 6 h, the level of cleaved caspase-3 was increased in dose dependent manner compared to untreated control. Combined treatment of METH and caffeine showed a significant increase in caspase-3 activation compared to cells treated only with METH (Figure 5.10). These results demonstrated that caffeine potentiate toxic effect of METH via increasing caspase-3activation.

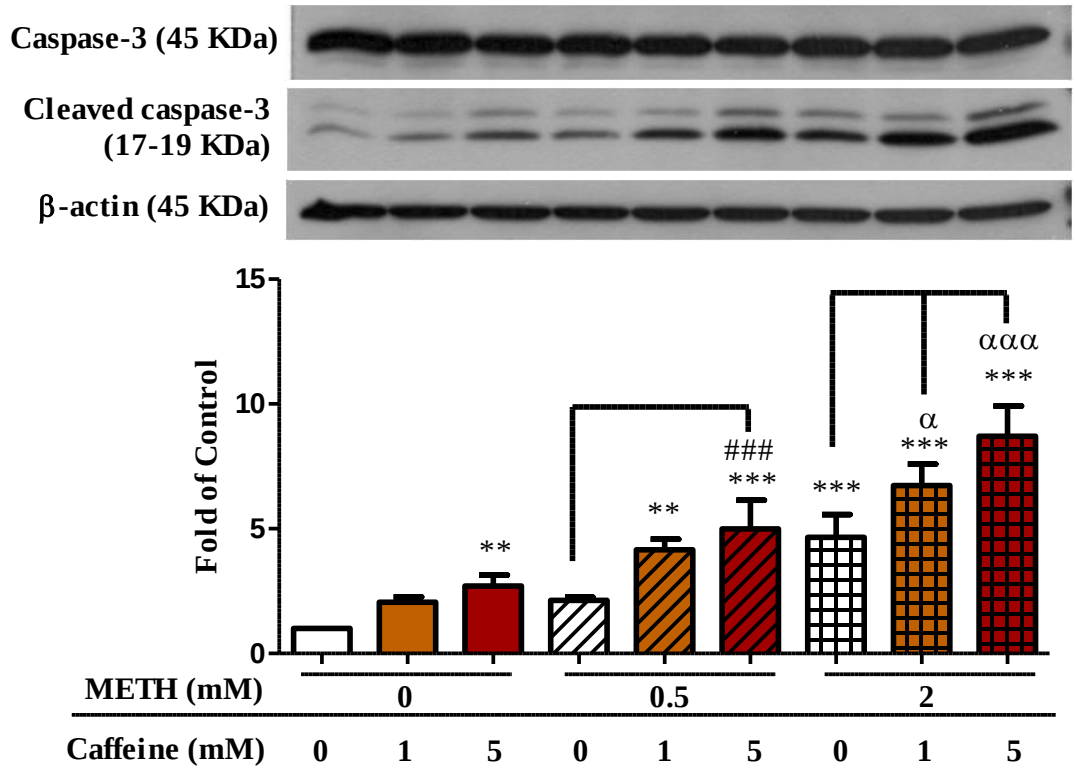


Figure 5.10 Effect of co-treatment between METH and caffeine on cleaved caspase-3 protein levels in SH-SY5Y cultured cells. Cells were treated with various condition of co-treatment for 6 h. The amount of cleaved caspase-3 was determined using Western blot analysis. β-actin was used as a loading control. The ANOVA was performed for statistical analysis ($^{**}p < 0.01$ and $^{***}p < 0.001$ compared with control, $^{###}p < 0.001$ compared with sample treated with METH 0.5 mM, $^{\alpha}p < 0.05$ and $^{\alpha\alpha\alpha}p < 0.05$ compared with sample treated with METH 2 mM).

### 5.5.2 Role of autophagy on the potentiating effect of caffeine in METH-induced neurotoxicity

To determine the role of autophagy on the potentiating effect of caffeine on METH- induced neurotoxicity in dopaminergic neuronal cell line, SH-SY5Y cells were pretreatment with an autophagy inhibitor, 3-methyladenine (3-MA), for 1 h before exposed to combination treatment of METH and caffeine.

5.5.2.1 Effect of 3-MA on cell viability in the combination treatment of METH and caffeine.

SH-SY5Y cells were exposed to various concentrations for combined treatment of METH and caffeine for 24 h with or without pretreatment with 3-MA at 5 mM for 1 h. After incubation, the cell viability was determined using MTT assay. The result showed that pretreatment with 3-MA prior to combined treatment of METH and caffeine increased cells toxicity compared to the combined treatment of METH and caffeine without pretreatment.

Effect of 3-MA on cell viability in the combined treatment of METH and caffeine was showed on table 5.1. Inhibition of autophagy by pretreatment with 3-MA in the combined treatment of METH and caffeine decreased cell viability compared to combined treatment of METH and caffeine alone (figure 5.11). These suggested the protective role of autophagy on the potentiating effect of caffeine on METH- induced neurotoxicity in dopaminergic neuronal cell line.

Table 5.1 Effect of 3-MA on cell viability in the combination treatment of METH and caffeine.

| Treatment | without 3-MA | | 3-MA | |
|---|---|---|---|---|
| CAF 1 mM | 99.48 | ±5.14 | 92.01 | ±1.60 |
| CAF 5 mM | 67.44 | ±1.50 | 67.24 | ±3.29 |
| METH 0.5 mM | 102.6 | ±6.47 | 83.58 | ±2.16 |
| METH 0.5+CAF1 mM | 86.13 | ±2.98 | 61.91 | ±6.09 |
| METH 0.5+CAF5 mM | 56.33 | ±2.30 | 42.25 | ±10.2 |
| METH 2 mM | 63.64 | ±7.23 | 48.94 | ±0.65 |
| METH 2+CAF1 mM | 49.58 | ±1.70 | 39.39 | ±6.86 |
| METH 2+CAF5 mM | 37.88 | ±2.74 | 29.38 | ±1.13 |

The results are expressed as mean ± SEM of untreated control.

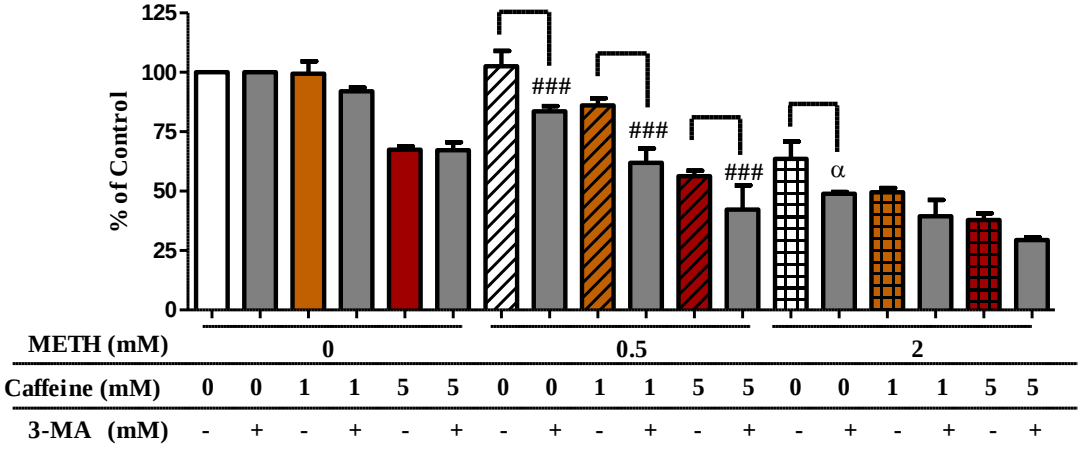


Figure 5.11 Effect of autophagy inhibitor, 3-methyladenine (3-MA), on cell viability in SH-SY5Y cultured cells. SH-SY5Y cells were co-treated with METH and caffeine at various concentrations for 24 h with or without pretreated with 5 mM 3-MA for 1 h. Cell viability was determined using MTT assay. The results are expressed as mean ± SEM. The ANOVA was performed for statistical analysis ($^{###}p < 0.001$ compared with sample treated with METH 0.5 mM, $^{\alpha}p < 0.05$ compared with sample treated with METH 2 mM).

5.5.2.2 Effect of 3-MA on cleaved caspase-3 expression in the combination treatment of METH and caffeine.

The effect of 3-MA on caspase-3 expression in the combination treatment of METH and caffeine was determined by western blot analysis. Induction of apoptosis on caffeine (5 mM) and combination treatment of METH (2 mM and caffeine (5 mM) in SH-SY5Y cells was determined by the amount of cleaved caspase-3 protein levels. The results showed that blocking autophagy in SH-SY5Y cells with 3-MA increased the amount of cleaved caspase-3 when compared to sample without 3-MA treatment (Figure 5.12). This data confirmed that the increase in autophagic flux effect of caffeine help protect cells from apoptosis cell death.

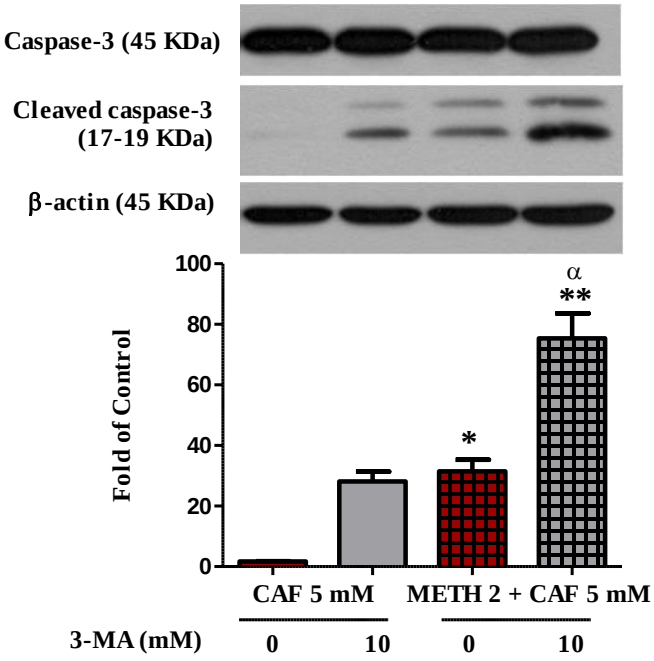


Figure 5.12 Effect 3-MA induced induction of cleaved caspase-3 protein levels in SH-SY5Y cultured cells treated with caffeine. SH-SY5Y cells were treated with 5 mM caffeine and co treated with Meth 2 and caffeine 5 mM for 6 h with or without pretreated of 10 mM 3-MA for 1 h. The amount of cleaved caspase-3 was determined using Western blot analysis. β-actin was used as a loading control. The ANOVA was performed for statistical analysis ($^{*}p < 0.05$ and $^{**}p < 0.01$ compared with untreated control, $^{\alpha}p < 0.05$ compared with sample treated with METH 2 mM).

## 5.6 Effect of METH and caffeine on morphology of SH-SY5Y cells

Cultures of SH-SY5Y human neuroblastoma cells were treated with METH (0.5 and 2 mM), caffeine (1 and 5 mM), and combined treatment of METH and caffeine for 6 hours. Cells morphology was observed with light microscope and captured with digital camera. The result showed that caffeine at 1 and 5 mM were slightly alter cell morphology compared to untreated control. METH at 0.5 mM did not alter SH-SY5Y cell morphology compared to untreated control while METH at 2 mM induced massive cytoplasmic vacuolization and caused cell partially detached from culture dish. Combined treatment of caffeine 1 and 5 mM with 2 mM METH treatment decreased cytoplasmic vacuolization and increased cell detachment in a dose dependent manner compared to METH 2 mM treatment alone (figure 5.13).

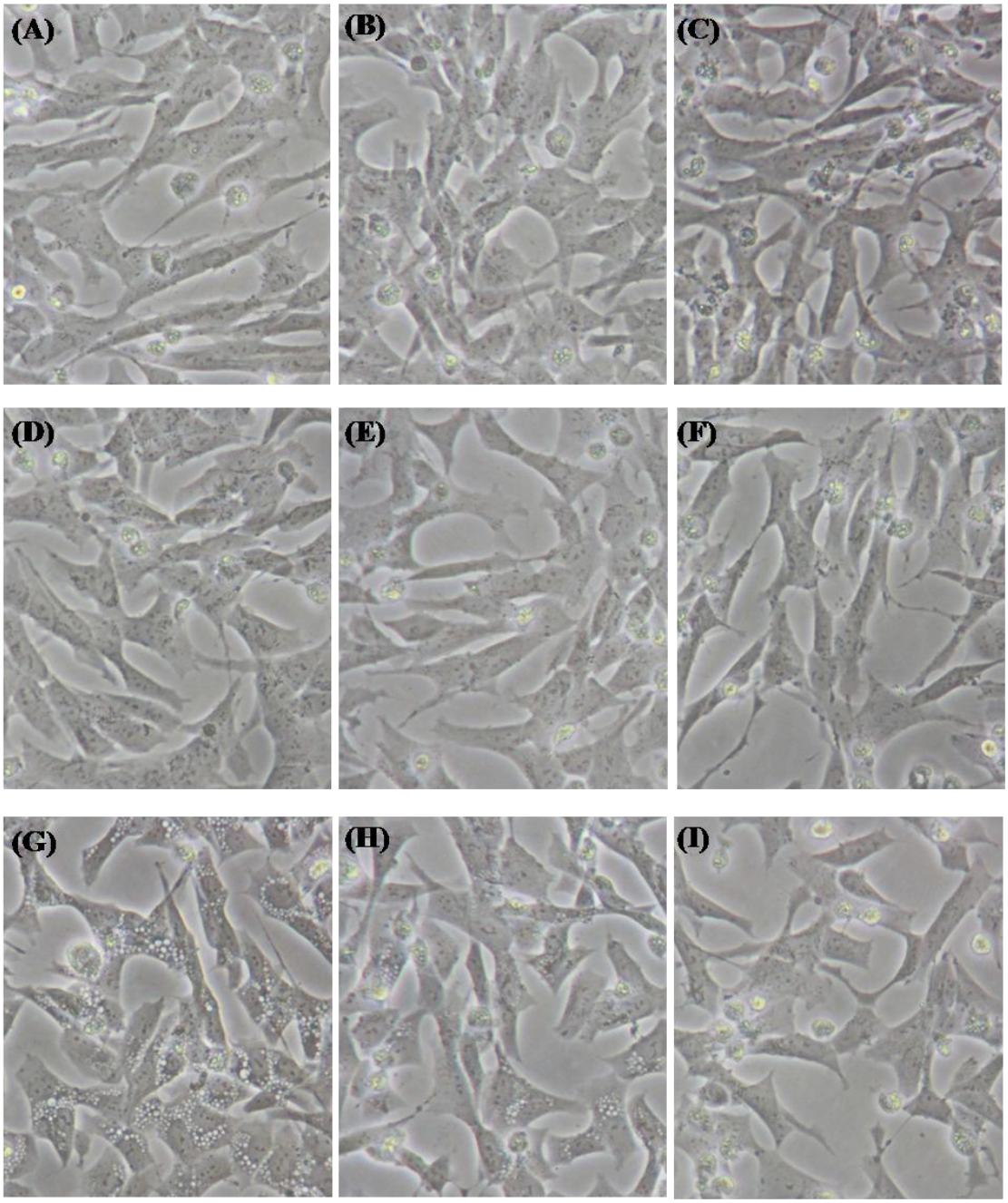


Figure 5.13 Morphology of SH-SY5Y neuroblastoma cells. The cultures was observed at 6 hours with (A) untreated control, cells treated with (B) caffeine 1mM, (C) caffeine 5 mM, (D) METH 0.5 mM, (E) METH 0.5 and caffeine 1 mM, (F) METH 0.5 and caffeine 5 mM, (G) METH 2 mM, (H) METH 2 and caffeine 1 mM, and (I) METH 2 and caffeine 5 mM.